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THE EFFECT OF GOITROGENIC AND OTHER REDUCING AGENTS ON INACTIVATED THYROTROPIC HORMONE EXTRACT[1]

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IN A PREVIOUS paper (Albert et al., 1946) we have shown that treatment of a thyroid stimulating hormone (TSH) extract with elementary iodine resulted in precipitation of most of the proteins of the extract. The brown iodinated precipitate was observed to have lost 95 to 100 per cent of its original thyrotropic activity. When the precipitate was triturated in an excess of acetone, 60 per cent of the iodine was removed, leaving a buff-colored protein residue containing the original thyrotropic potency.

The conversion of inactivated hormonal material to activity has been accomplished in other ways. When the iodinated TSH extract precipitate was triturated with solutions of 2-thiouracil, 6-N-propyl thiouracil, aminothiazole, 5-amino-2-mercaptothiadiazole (T.C. 68), 3-phenylaminomethyl-2-mercaptothiazoline (T.C. 105) and potassium thiocyanate the precipitate immediately dissolved yielding a water clear solution containing in varying degree the original thyrotropic action. Since all of these goitrogens are reducing compounds of varying intensity, and since restoration of hormonal activity was accomplished simultaneously with reduction of iodine loosely bound to the protein precipitate, other reducing but non-goitrogenic agents were studied. Ascorbic acid and sodium thiosulfate were found to restore the activity and inactivated hormonal material simultaneously

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with reduction of the bound iodine and reversal of the insolubility of the proteins. However, under identical conditions, NaOH was found to decolorize the iodine and to dissolve the precipitate without restoration of activity.

METHODS

The methods of assay of TSH, described in our previous paper, have been used without change. TSH extract (Antuitrin T, Parke, Davis and Company) was inactivated by addition of accurate amounts (30 Junkmann-Schoeller units) to a series of 50 ml. centrifuge tubes containing 30 ml. dilute Lugol's solution. After mixing, the tubes were maintained at room temperature for two hours, during which time a brown precipitate formed. The supernatant fluid was decanted after centrifugation and excess iodine removed from the walls by washing with distilled water while rotating the tubes on an incline. The precipitate was triturated to an even suspension in 1 ml. distilled water, a drop of starch (1 per cent) added as indicator, and the various reducing agents added dropwise until the color just completely disappeared. The resulting water clear solution was diluted to 45 ml., of which 1 ml. was injected once daily for three days into each of 10–15 one-day old chicks, each animal thus receiving the equivalent of 2 units of the original TSH extract. Control experiments consisted of: (a) animals not injected, (b) animals given 2 units untreated TSH extract, (c) animals given 2 units equivalent of iodinated TSH extract, (d) animals given the same amount of reducing compounds as used in the reduction of iodinated hormone extract, (e) animals given the same amount of iodine present in the equivalent of 2 units iodinated extract (200 μg. iodine), (f) animals given both 200 μg. iodine and the same amount of reducing compounds, and (g) animals given both the same amount of reducing compound and 2 units of untreated TSH.

The preparation of dilute Lugol's solution was described in our previous report. Solutions of anti-thyroid compounds were made by adding 1 gm. of the substance in 100 ml. distilled water with repeated shaking at room temperature. Soluble compounds (KSCN, $Na_2S_2O_3$, ascorbic acid) were used as such (10 mg. per ml. solution). The remaining agents were used as saturated solutions after removal of undissolved material.

RESULTS

The effect of eight reducing agents on the iodine-inactivated TSH extract is given in table 1. Assuming that 5 per cent TSH activity remained in the iodinated precipitate (actually none can be detected at the dose level employed), it was calculated[2] that the activity of the iodinated material after exposure to reducing compounds was be-

[2] This calculation was made by reading off the experimental values (average thyroid weight) shown in tables 1–3 in terms of units from Figure 1 of our previous paper. The percentage difference between the control value (iodinated TSH extract assumed to be 1/20 unit instead of 0) and the units of any experimental run was computed. The values for cell heights and thyroidal iodine concentration cannot be treated in this fashion, as explained previously, but it can be noted that they usually follow the same trend indicated by the thyroid weights; i.e., the more intense the thyrotropic action, the greater the thyroid weight and cell height, but the lesser the iodine concentration.

TABLE 1. EFFECT OF GOITROGENS AND OTHER REDUCING AGENTS ON IODO-TSH

Assay no.	Substances per chick	Thyroid wt. mg.	Thyroid I μg./mg.	M.C.H.* micra
157	None	2.95	1.87	4.10
158	2 units TSH**	7.02	0.11	9.12
159	2 units Iodo TSH**	3.40	0.90	3.94
161	2 units Iodo TSH and Thiouracil (0.31 mg.)	5.90	0.62	6.91
162	2 units Iodo TSH and Ascorbic Acid (0.268 mg.)	6.38	0.48	7.56
163	2 units Iodo TSH and $Na_2S_2O_3$ (0.66 mg.)	5.22	0.53	8.45
164	2 units Iodo TSH and KSCN (13.3 mg.)	5.86	0.14	8.31
165	2 units Iodo TSH and TC 68 (0.22 mg.)	6.25	0.21	8.83
166	2 units Iodo TSH and TC 105 (0.046 mg.)	5.07	0.41	6.88
167	2 units Iodo TSH and NaOH***	3.27	1.01	4.43
168	2 units Iodo TSH and Propyl Thiouracil (1.14 mg.)	6.89	0.29	7.85
169	2 units Iodo TSH and Aminothiazole (1.08 mg.)	5.11	0.21	6.78

* M.C.H. = mean acinar cell height.

** The terms TSH and Iodo TSH are abbreviations respectively for thyrotropic extract and iodine treated thyrotropic extract.

*** 0.01 N NaOH was added dropwise until decoloration occurred (final pH of clear solution = 6.5).

tween 1000 and 2000 per cent greater than before reduction. This was observed for all agents studied except NaOH, which induced no change in hormonal activity. The restoration of activity was not due to any effect of the reducing compounds on the chick thyroid, since when given in the same dosage employed in table 1, they were without goitrogenic (or thyrotropic) effect (table 2). Iodine alone (200 μg.), stated in our previous report, was without effect on the thyroid except to increase slightly the thyroidal iodine concentration (an anti-thyrotropic action). When iodine was given concurrently with the reducing compounds, no effect on the thyroid was detectable (table 3).

The results of these data including control experiments (a–f) showed that the reactivation of the iodinated TSH extract was due to some interaction between the reducing agents and the iodinated pre-

TABLE 2. EFFECT OF GOITROGENS AND OTHER REDUCING AGENTS ON THE CHICK THYROID

Assay no.	Substances per chick*	Thyroid wt. mg.	Thyroid I μg./mg.	M.C.H. micra
170	Aminothiazole	3.06	1.31	3.90
171	Propyl Thiouracil	3.14	1.20	3.95
101	Thiouracil	3.36	1.60	3.83
102	Ascorbic Acid	3.25	1.45	3.76
103	KSCN	4.00	0.97	3.88
104	$Na_2S_2O_3$	4.10	1.31	3.80
105	TC 68	3.40	1.15	3.94
106	TC 105	3.30	1.20	3.94
90	None	2.70	0.98	3.93

* Amounts of reducing agents used same as given in table 1.

cipitate and not to the reducing agents themselves or to their interaction with any one of the other two components (TSH and iodine) of the system. This was further supported by the results of control experiment (g). This experiment was included to test an assumption that the reactivation might actually be due to some augmenting action of the reducing compounds on a small portion of TSH extract escaping inactivation during iodine treatment (assumed to be 5 per cent of the original activity). The results of this and related experiments, which will be described separately because of certain physiological significance, showed that augmentation did occur, but at most,

TABLE 3. EFFECT OF GOITROGENS AND REDUCING AGENTS PLUS IODINE ON THE CHICK THYROID

Assay no.	Substances per chick	Thyroid wt. mg.	Thyroid I μg./mg.	M.C.H. micra
195	None	3.19	1.10	3.86
201	200 μg. Iodine* & Thiouracil**	3.34	0.97	3.97
202	200 μg. Iodine & Ascorbic Acid	3.52	0.89	3.99
203	200 μg. Iodine & $Na_2S_2O_3$	3.48	1.40	3.90
204	200 μg. Iodine & KSCN	4.00	1.20	3.99
205	200 μg. Iodine & TC 68	2.94	0.97	4.02
206	200 μg. Iodine & TC 105	3.20	1.38	3.95
207	200 μg. Iodine & Amirothiazole	3.20	1.46	3.89
208	200 μg. Iodine & Propyl Thiouracil	3.64	1.07	4 18

* Iodine solution used was dilute Lugol's solution, 1 ml. = 10 mg. total iodine, $\frac{1}{3}$ of which was elementary and $\frac{2}{3}$ iodide.

** Amounts of reducing agents used same as given in table 1.

amounted to a 60 per cent increment in thyrotropic potency. Since this augmentation represented only 3 to 6 per cent of the overall change (table 1) in potency after reduction, it was considered that the the effect of reduction was some chemical action on the inactivated hormonal material.

SUMMARY

The loss of thyrotropic activity, occurring during exposure of a pituitary extract to iodine, was restored in varying degree by treatment of the iodinated hormonal material with various goitrogenic and non-goitrogenic reducing compounds.

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AUGMENTATION OF THYROTROPIC POTENCY BY GOITROGENS[1]

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IN THE PRECEDING paper, it was shown that the reactivation of iodine-treated (inactivated) thyrotropic extract was accomplished by several goitrogenic and non-goitrogenic reducing compounds. It was also observed, in control experiments, that exposure of known amounts of active thyrotropic (TSH) extract to a variety of reducing agents resulted in enhancement of the thyroid stimulating activity of the extract. The augmenting effect produced by goitrogenic agents was found on further investigation to be much greater than that induced by the non-goitrogenic compounds when calculated in terms of percentage augmentation per mg. reducing agent. Furthermore, it was found that the augmenting action was not dependent on the continued presence of the augmenting agent during the bio-assay procedure, since the agents were presumably removed from the hormonal material by prolonged dialysis or the hormonal material presumably removed from the agents by precipitation with ammonium sulfate.

MATERIALS AND METHODS

The TSH extract, the preparation of solutions of the various reducing compounds, and the method of bio-assay have been described in our previous reports (Albert et al. 1946, 1947). In the present experiments 15 Junkmann-Schoeller units TSH extract (6 mg. protein) was quantitatively added to a series of 50 ml. centrifuge tubes containing the various reducing substances. The amounts of these substances were the same as previously used and given in table 1. The mixtures were diluted to 25 ml. with distilled water and were either left at room temperature for one-half hour or incubated at 37.5° C. for two hours. Controls consisted of the same amount of TSH made up to 25 ml. with distilled water and treated in exactly the same fashion. After incubation or standing, distilled water was added to each tube to 45 ml. and assays performed. Thus each of a group of 10 to 15, one-day old chicks, received a total of 1 unit TSH.

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In another series of experiments 2 volumes of saturated ammonium sulfate were added to each tube after incubation and the tubes left overnight at 5° C. After centrifugation the supernatant fluid was discarded and the white precipitate dissolved in water and diluted to 45 ml. for assay. A third series of experiments consisted of transferring, after incubation, the contents of each tube quantitatively to ½-inch diameter cellophane bags (Visking Company). The bag contents were dialyzed for 48 hours against cold running tap water (15–18° C.), after which the mixtures were transferred, as quantitatively as possible, to vessels and made up to 45 ml. for assay.

DISCUSSION OF RESULTS

The augmenting effect of goitrogenic agents on the thyrotropic activity of the pituitary extract is illustrated by two separate experiments (table 1). It can be noted in series 172–176 that all reducing compounds induced augmentation except ascorbic acid, which apparently caused destruction of hormonal material. In a larger series, 177–186, the same relations were obtained.

Two points of consideration arise from the values given in table 1, the first of which is a comparison of the three values (thyroid weight, thyroidal iodine concentration and mean acinar cell height) used to measure thyrotropic activity. As is well known, thyroid weight and cell height are directly, and iodine concentration inversely, proportional to TSH within certain limits of dosage. However, for each value the dosage range is different; thyroidal iodine concentration being most sensitive (i.e., most responsive to small dosages of TSH), followed by cell height and thyroid weight respectively. In the dosage employed for bio-assay (1 unit), the values for iodine concentration and mean cell height are asymptotic or becoming so, whereas the values for thyroid weight are well within the sensitive portion of the dose-response curve. In spite of this, there is surprisingly good agreement among the three values, considering that only one iodine analysis was done on the pooled chick thyroids. When treatment of TSH extract resulted in depression or elevation of thyrotropic activity as determined by thyroidal weight, the values for mean cell height and thyroidal iodine concentration, irrespective of the intensity of their changes, varied in the same direction. Certain exceptions, however, were noted. $Na_2S_2O_3$ induced slightly heavier thyroids and larger cell heights than the controls, but the thyroidal iodine concentration was higher rather than lower than the controls. Two of the criteria of thyrotropic activity thus indicated slight augmentation, whereas the third indicated slight depression. It can be noted also from table 1 that the same was true, in a reverse direction, in the case of mercaptothiadiazole (TC-68), which induced slight depression of activity as measured by weight and cytology, but augmentation by iodine analysis. Because the iodine and mean cell height values are in the asymptotic portion of the dose-response curve (i.e., their ability to discriminate among small changes in dosage is low to nil), calculations were

made entirely from the values of thyroidal weights. The methods for calculation of percentage change have been previously described.

The second point of consideration (the error of the bio-assay) deserves detailed comment. It can be noted (table 1) that 1 unit TSH control (assay No. 176) induced a thyroidal weight of 5.62 mg. whereas in another shipment of chicks several weeks later 1 unit TSH (assay No. 178) prepared from the same batch of extract induced a weight of 6.25 mg. This variation in the TSH standard represents

TABLE 1. AUGMENTATION OF THYROTROPIC HORMONE BY GOITROGENS

Assay no.	Agents per chick	Thyroid wt. mg.	Thyroid I μg./mg.	M.C.H. micra	Per cent change
177*	None	3.65	1.20	4.11	—
178	1 unit TSH	6.25	0.25	7.77	0
179	1 unit TSH & Thiouracil (0.31 mg.)	7.55	0.11	8.37	+46
180	1 unit TSH & Ascorbic Acid (0.268 mg.)	4.05	0.46	5.98	−83
181	1 unit TSH & $Na_2S_2O_3$ (0.66 mg.)	6.48	0.31	8.27	+10
182	1 unit TSH & KSCN (13.3 mg.)	6.80	0.11	9.06	+20
183	1 unit TSH & TC 68 (0.22 mg.)	5.81	0.09	7.63	−17
184	1 unit TSH & TC 105 (0.046 mg.)	7.22	0.26	9.04	+37
185	1 unit TSH & propyl thiouracil (1.14 mg.)	6.60	0.08	9.11	+11
186	1 unit TSH & Aminothiazole (1.08 mg.)	6.87	0.17	9.02	+23
172**	1 unit TSH & Thiouracil***	6.56	0.03	—	+50
173	1 unit TSH & Ascorbi Acid	4.69	0.30	—	−58
174	1 unit TSH & KSCN	7.09	0.01	—	+78
175	1 unit TSH & $Na_2S_2O_3$	6.30	0.08	—	+40
176	1 unit TSH	5.62	0.14	—	0

TC 68 = 5 amino-2-mercaptothiadiazole.
TC 105 = 3 phenylaminomethyl-2-mercaptothiazoline.
177–186 series = *(mixtures incubated 2 hours at 37.5° C.).
172–176 series = ** (mixtures maintained at room temperature (24° C.) for ½ hour).
*** = amounts of agents used same as corresponding amounts in series 177–186.

probably the overall inherent and aperiodic variations of the assay and emphasizes again that there is no fixed standard of response to apparently the same dosage of hormone. If assays are to be compared, a control standard must be run for each shipment of chicks, and the results computed as percentage deviation from this control standard. These percentage deviations[2] can then be compared with similar ones obtained at another time, because the slopes of the dose-response

[2] The percentage changes in units of the experimental values over the control dose of TSH administered are greater, of course, when the standard in units is lower than when the standard is higher. However, lacking a fixed standard such as an International Standard preparation of TSH, this method of comparison, though admittedly inexact, is the only convenient way of handling groups of similar material. Since we are not at this time interested in exact comparative values, which can be obtained only by testing several dosages of the standard versus several dosages of each of the experimental mixtures, the more elaborate methods of calculation were not undertaken.

curves are similar, but the actual weights obtained at different intervals cannot be compared.

A summary of all the results obtained is given in table 2 which shows in addition to the values listed in table 1 the data for the experiments in which the components of the mixture were presumably separated prior to assay. Despite the small quantities of protein handled, the data of the various experiments are in fairly good agreement. For five of the agents, all of the results obtained were in the same direction, irrespective of the magnitude of the change. The three remaining agents showed some deviations; i.e., $Na_2S_2O_3$ induced 4 plus and 1 minus change, TC 68 and TC 105 both induced one negative change and two positive changes in thyrotropic potency. The explanation of these deviations was not clear. The average values, comprising over 500 animals, reflect the general trend under all four types

TABLE 2. SUMMARY OF AUGMENTATION OF TSH

Agents	1	2	3	4	5	Average change per cent	Average per cent change, per mg.
Thiouracil	+50	+46	+ 3	+47	+49	+39	+126
Ascorbic Acid	−58	−83	−91	—	—	−77	−270
KSCN	+78	+20	+30	+10	+72	+42	+ 3
$Na_2S_2O_3$	+40	+10	+30	− 8	+ 4	+15	+ 22
TC 68		−17	+87	+100	—	+56	+254
TC 105		+37	+91	−29	—	+33	+710
Propyl Thiouracil		+11	—	—	+20	+15	+ 13
Aminothiazole		+23	+78	+37	+75	+43	+ 40

1 = Mixed at room temperature (table 1).
2 = Incubated at 37.5° C., 2 hours (table 1).
3 = Incubated, then dialyzed.
4 = Incubated, then dialyzed.
5 = Incubated, then TSH precipitated by 2 volumes $(NH_4)_2SO_4$.
— = Data not obtained because of difficulty in centrifugation, or dialysis. Values are in per cent change over respective standards.

of experiments and are probably valid for each agent, despite the indicated deviations. Since the accuracy of the bio-assay is not known with certainty and may be no greater than ± 20 per cent, the average values for thiouracil, ascorbic acid, KSCN, TC 68, TC 105 and aminothiazole seem reasonably outside the apparent error of assay. The values for $Na_2S_2O_3$ and propyl thiouracil, however, could be regarded within experimental error.

The chief conclusion that augmentation of TSH activity was induced by certain reducing agents seems obvious from table 2. Furthermore, the goitrogenic compounds were much more effective in this regard than non-goitrogenic compounds. Moreover, this effect was induced by the goitrogens in doses which when given alone had no effect on the thyroids of test animals, as could be ascertained by the methods employed. The effect was still evident after the goitrogen

was presumably removed prior to bio-assay. It would appear that exposure of TSH extract to such agents had resulted in some alteration leading to enhanced thyrotropic activity. That reduction per se did not explain these results completely was evident from the observation that ascorbic acid, a strong reducing agent, destroyed TSH activity instead of enhancing it.

When the average augmentation was computed on a mg. basis of respective agent (table 2), an interesting correlation of the eight compounds studied was possible. Aminothiazole, thiouracil, TC-68 and TC-105 in that order showed increasing augmenting ability, a sequence which parallels their goitrogenic ability in the rat (Astwood, 1945). Propyl thiouracil was the exception. KSCN had no augmenting ability when calculated in this fashion, and it is interesting that the effect of this goitrogen, unlike those of the mercaptans and thiourylene series is more dependent on the level of iodine intake (Astwood, 1945). This correlation may be purely fortuitous. Further study of other compounds, varying in goitrogenic ability, will be necessary before any conclusions in this regard can be drawn.

SUMMARY

Exposure of a pituitary extract to various goitrogenic compounds resulted in augmentation of its thyrotropic activity. The augmenting effect was not abolished when the goitrogens were presumably removed from the hormonal material prior to bio-assay.

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GONADAL HORMONES AND THE CONTRACTILITY *IN VITRO* OF THE VAS DEFERENS OF THE DOG

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ALTHOUGH the motility of the uterus of the bitch has been the subject of many papers there is none dealing with the endocrine control of the contractility of the male organs. Waddell (1916) found the quiescent vas deferens of the dog to react with tonic and rhythmic contractions on addition of various drugs to the perfusion bath. Macht (1917), using longitudinal strips as well as rings from the organ, was unable to observe any automatism in those preparations. *In vivo* experiments made by Perutz & Merdler (1924) showed that the dog's vas deferens displayed contractions not only after intravenously injected drugs but also upon stimulation of the vagus, of the nasal mucosa, and following asphyxia. Iwaki (1930) did not observe automatism *in vitro* in any of the four vasa deferentia from three dogs; he called attention to the delayed effect of the drugs studied by him.

Using the same technique employed in our previous works about the hormonal regulation of the motility and pharmacological reactivity of the male genitals, we shall analyze in the present paper the behavior *in vitro* of the vas deferens of the dog.

MATERIAL AND METHODS

We used 7 normal and 29 castrated mature dogs of mixed strains weighing 5 to 12 kgr. They were kept in large cages with meat as the main food. Castration was performed 33 to 420 days earlier under ether anesthesia, after scrotal incision; a small portion of the tail of the epididymis close to the vas deferens was left in place. The group of castrates included 11 untreated and 18 treated animals; testosterone propionate was administered to 6, estradiol benzoate to 8, and sesame or arachis oil to 4. The treatment consisted in subcutaneous injections, two or three times a week, of the hormone in oil[2] or in implantations (under the dorsal skin) of the hormonal pellets weighing 10 to 15 mgr. The individual data are given in table 1.

The vas deferens was removed under ether anesthesia through a suprapubic incision, dissected, stripped of all surrounding connective tissue, and divided into distal and proximal segments 6 to 8 cms. long. Both of them were studied either on the same day or one of them was kept in the refriger-

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[2] Supplied as testosterone propionate and estradiol benzoate through the courtesy of Ciba S.A. (Perandren), Schering S.A. (Testoviron and Progynon) and Organon S.A. (Neohombreol and Dimenformon).

ator and tested the next morning. The organs were transferred to a beaker containing 60 to 80 cc. of oxygenated Locke's solution at 38–39° C. Longitudinal contractions were registered with an amplification index of 6 and a load of 2 grs. At the same time we tested in the same beaker 2 or 3 vasa deferentia from animals under different hormonal conditions. Forty to 60

TABLE 1. TREATMENT OF DOGS

Dog number[1]	Final weight (kgr.)	Days since castration	Treatment and total dosage
		Normals	
1B	6.6		No treatment
20A	20.2		No treatment
26A	7.5		No treatment
28A	10.8		No treatment
30A	8.1		No treatment
39B	7.0		No treatment
40A	12.0		No treatment
		Castrates	
2B	8.0	319	No treatment
8A	10.0	34	No treatment
10A	17.0	52	No treatment
13B	6.3	270	No treatment
14B	6.3	257	No treatment
18A	12.0	148	No treatment
22A	6.4	249	No treatment
25A	6.7	264	No treatment
27A	17.0	75	No treatment
55B	5.0	357	No treatment
56B	6.0	357	No treatment
17A	10.0	157	Sesame oil
31B	7.2	35	Arachis oil
37B	8.0	377	Arachis oil
57B	5.0	352	Arachis oil
		Testosterone propionate treated castrates	
12A	9.0	34	20 mgr. implanted +40 mgr. injected
12B	6.6	262	81 mgr. implanted
16A	8.5	73	230 mgr. injected
18B	9.8	268	81 mgr. implanted +60 mgr. injected
19A[2]	10.8	33	200 mgr. injected
35B	7.2	35	20 mgr. implanted +140 mgr. injected
		Estradiol benzoate treated castrates	
4B	6.5	258	32 mgr. injected
5B	6.3	262	32 mgr. injected
13A	10.0	34	10 mgr. implanted +15 mgr. injected
14A	10.3	77	45 mgr. injected
15A	8.5	73	33 mgr. injected
34B	7.7	35	15 mgr. injected
36B	8.3	377	27 mgr. injected
38B	9.0	420	32 mgr. injected

[1] A—animals supplied by the Municipality. B—dogs bred at our Institute. Dogs 36, 37, 38 and 39; 55, 56 and 57; 4 and 5; 12, 13 and 14 are littermates.
[2] Previously employed as normal.

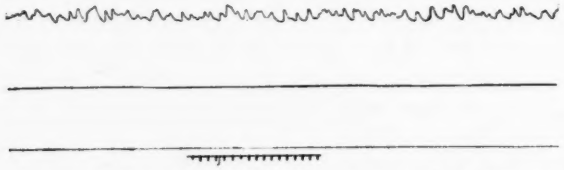
minutes after the onset of the experiment we started a study of the effects of drug solutions added to the nutritive bath to the final concentrations given below.

After the first experiment, lasting 2 to 4 hours, the organs were removed from the bath, cooled in fresh Locke's solution and placed in a refrigerator. After varying intervals of days, further experiments on the same strips repeatedly were made under the conditions described above.

RESULTS

The contractility and the excitability of the organs were estimated by the incidence, frequency and amplitude of the spontaneous contractions (automatism) and by the type, intensity and delay of responses to the pharmacological agents.

Automatism. The incidence of spontaneous contractions during the first hour after placing the organs into the perfusing bath, was observed in organs from 10.3% of the untreated castrated animals and in organs from 68.8% of the castrates treated with estradiol benzoate. The frequency and amplitude of contractions varied considerably, the automatism being more obvious in vasa deferentia of



Simultaneous records of vasa deferentia of dogs. Time marks in every case, 5 seconds.
FIG. 1. Vasa deferentia of dogs, 35 days after bilateral castration. *Upper:* Dog 13A, treated with 25 mgr. of estradiol benzoate. *Center:* Dog 8A, untreated. *Lower:* Dog 12A, treated with 60 mgr. of testosterone propionate. Spontaneous contractions only exhibited by the vas deferens of the estradiol treated donor.

castrates treated with estradiol than in those of untreated castrates. Only one vas deferens of a testosterone implanted animal (12B) exhibited automatic movements.

Effects of drugs. The vasa deferentia taken from estradiol treated castrates were the most responsive to the different drugs assayed. In order of decreasing reactivity came the organs from untreated castrates, then those from testosterone treated castrates and those from normal dogs as least reactive.

Epinephrine hydrochloride (1:2,000,000–1:10,000,000). The vasa deferentia of donors from groups other than the normal reacted well to this drug. When the organs from normal or from testosterone treated castrated dogs exhibited a response to epinephrine, the latent period was generally greater than in other cases. In all instances the contractions were mainly of the tonic rhythmic type especially in the organs taken from estradiol treated castrates. Some tests showed the inactivity of epinephrine after a previous addition of ergotamine tartrate (1:600,000).

Acetylcholine hydrochloride (1:20,000–1:80,000). At this concentration this drug usually was a weak stimulant. A higher incidence of tonic or tonic-rhythmic response was obtained with organs taken from untreated castrates and estradiol treated castrates. Previous addition of eserine (1:800,000) may sensitize the vasa deferentia to the subsequent action of acetylcholine.

Histamine dihydrochloride (1:800,000–1:20,000,000) had a stimu-

lating action and provoked tonic or rhythmic contractions in the organs taken from untreated castrates or from estradiol treated castrates. There was practically no response by the vasa deferentia extirpated from normal or testosterone treated dogs.

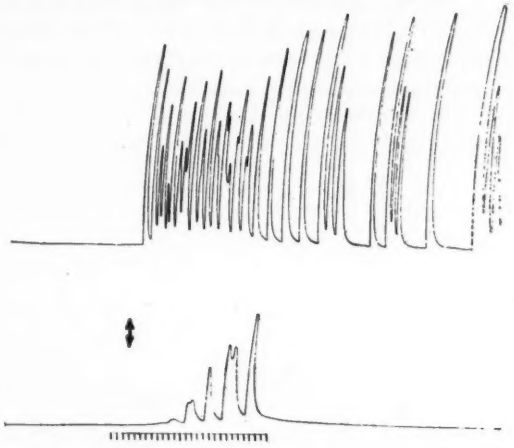


FIG. 2. Effect of epinephrine hydrochloride (1:10 millions). *Upper:* Dog 14A, 77 days after bilateral castration and treated the last 35 days with 45 mgr. of estradiol benzoate. *Lower:* Dog 19A, 33 days after bilateral castration and treated with 200 mgs. of testosterone propionate.

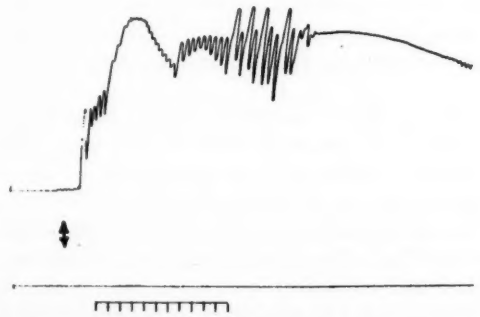


FIG. 3. Effect of histamine dihydrochloride (1:800,000) upon the vasa deferentia of dogs 73 days after bilateral castration. *Upper:* Dog 15A, treated with 33 mgr. of estradiol benzoate. *Lower:* Dog 16A, treated with 230 mgr. of testosterone propionate. The organs were previously maintained for 3 days at low temperature.

Ephedrine hydrochloride (1:20,000–1:40,000). The incidence of response, predominantly of the tonic-rhythmic type of contractions was greatest in the organs obtained from castrates or estradiol treated castrates. The vasa deferentia of normal dogs did not react; and those of testosterone treated castrates, after a long latent period, exhibited mainly tonic effects.

Pilocarpine hydrochloride (1:10,000–1:60,000). Contractions of mixed type were exhibited only by organs taken from untreated castrates and estradiol treated castrates.

Cocaine hydrochloride (1:15,000–1:30,000). Responses, mainly of a mixed type, were recorded for the organs taken from estradiol treated castrates. The latent period observed in the response to epinephrine by the organs from testosterone treated castrates may be shortened by a previous addition of cocaine.

Pituitrin (2–4 i.u. in 60 cc. of Locke). No response was exhibited by the organs of normal or testosterone treated animals. In the other

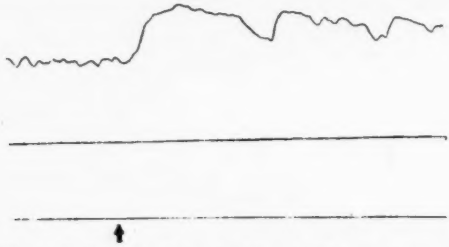


FIG. 4. Effect of pituitrin (2 i.u. in 80 cc.). The same organs as in Fig. 1. The drug was effective only upon the upper organ.

two groups the response was either tonic, rhythmic or mixed. In a few cases pitocin was also studied under the same concentration and with the same results.

Barium chloride (1:20,000–1:30,000) caused a mixed response in organs from estradiol treated castrates. The vasa deferentia taken from normal or testosterone treated castrates were poorly stimulated by this drug.

The responses to these different drugs, according to the type, intensity of reaction and group of donors, are summarized in table 2. A small number of additional tests were carried out with morphine (1:20,000), papaverine (1:40,000), nicotine (1:80,000) and atropine (1:40,000). Papaverine and atropine behaved as inhibiting agents; nicotine and morphine stimulated the vasa deferentia of castrates and estradiol treated castrates.

Survival of the organs. The effects of drugs as described above were inferred from tests made either on the day of extirpation of the vasa deferentia or within 72 hours after removal of the organs. In the subsequent days of cold storage there was a progressive decrease of sensitivity to the drugs assayed. The vasa deferentia of estradiol treated castrates seemed to be least resistent. Reaction to epinephrine may be recorded until 16 days after the first experiment.

COMMENTS

From the foregoing experiments it is concluded that the contractility and the pharmacological excitability of the vas deferens of the

dog, as well as of the other species studied earlier, depends on the hormonal conditions of the donors. The less responsive organs came from normal dogs and the more reactive ones from estradiol treated castrates. The organs taken from untreated castrates were more reactive than those from normals. Nevertheless, it should be noted that, in this species, the increased excitability of the vas deferens after castration, does not seem as evident as in the rat, the rhesus monkey,

TABLE 2. SUMMARY OF THE EFFECTS OF DRUGS IN VITRO UPON VASA DEFERENTIA OF DOGS

Drugs	Total of tests performed	No effect	Inhibition	Oscillation	Predominant type and intensity								
					Tonic			Rhythmic			Mixed		
					Weak	Med.	Strong	Weak	Med.	Strong	Weak	Med.	Strong
A. Normals													
Epinephrine	37	16		3	2	1	3	1		1	1		9
Acetylcholine	14	12		2									
Histamine	4	4											
Ephedrine	12	12											
Cocaine	17	17											
Pilocarpine	1	1											
Barium chloride	8	8											
Pituitrin	6	6											
B. Castrates													
Epinephrine	80			2	15	8	19		1	5	4	9	17
Acetylcholine	30	10		2	11	3	1					3	
Histamine	20	8		2	3	2	4				1		
Ephedrine	27	4		6	2	1	3	1	1		2	2	5
Cocaine	30	15	6	3	3		2				1		
Pilocarpine	13	10		2									1
Barium chloride	28	9		7	2	1	5				1		3
Pituitrin	12	7			1						1	1	2
C. Testosterone-Castrates													
Epinephrine	43	12		1	4	2	6		1		7	8	2
Acetylcholine	16	10		1	5								
Histamine	10	9						1					
Ephedrine	17	7		2		1	4		1			2	
Cocaine	17	13		2					1			1	
Pilocarpine	12	12											
Barium chloride	12	11									1		
Pituitrin	7	6			1								
D. Estradiol-Castrates													
Epinephrine	55	1				4	5	1	11	7	5	10	11
Acetylcholine	19	2		2		1	1	6	2		2	3	
Histamine	14					1	2				1	3	7
Ephedrine	24				1				5	6		4	8
Cocaine	22	1	2	1				1	1		1	4	11
Pilocarpine	15	3		3							2	4	3
Barium chloride	16			1					3		1	5	6
Pituitrin	12			1	2	1	1	1	2	1			

and the cat. Thus the incidence of automatism which approached 80% for the vasa deferentia of castrated rats does not exceed 10.3% for the vas deferens of dogs under the same conditions. Also the inhibitory action of androgenic treatment upon the motility, unquestionable for those animals, was less obvious in the present experiments. It is possible, however, that in some of the animals, this is due to the administration of an insufficient amount of testosterone. It is clear, however, that the pharmacological excitability of the vas deferens of testosterone treated castrates was more comparable to the normal than to the castrate type. It was mentioned above that in the testosterone treated group spontaneous contractions were recorded only once. All this leads us to believe that, in the dog, as in the above named species, the androgens inhibit the motility of the vas deferens.

One might argue that the high excitability of the vas deferens of estradiol treated dogs would depend on the increase of the number of smooth fibers of the ductus as a consequence of the known morphogenetical action of the estrogens. In contrast with this, we have found that the atrophic vasa deferentia of castrated dogs were more excitable that those of normal ones.

The excitability of the vas deferens of the normal dog is weaker than that, for instance, of the rat under the same conditions. Perhaps this hypoexcitability is a phenomenon connected with the ejaculation type of the two species.

We do not know which modifications of the vas deferens musculature account for the higher excitability when taken from castrates or from estradiol treated castrates. The graft of the vas deferens of the rat (Martins, Valle & Porto, 1940) and of the cat (Martins, Valle & Porto, 1941) in the gastric wall neither suppressed nor modified the differences of behavior which depend on hormonal balance. The transplanted organs behaved as if they were taken from their normal site. Thus the hormonal control of the motility of the male genital musculature seems to be exerted independently of nervous connections in harmony with the views of Kaminester & Reynolds (1935) for the uterus of the rabbit. It remains to be proved whether this is also the case for the dog.

Another question deserving comment here is the comparison between the effects of epinephrine and pituitrin upon the vas deferens of the dog, and the action of the same agents upon the uterus of the bitch. In a review of this subject, Reynolds (1939) quotes the view of several authors, among them Athias (1921), who conclude that the uterus of the bitch, like that of the cat, reacts to epinephrine with contraction when taken from pregnant animal and relaxes in normal circumstances. As regards the vas deferens the inversion of the epinephrine action under changed hormonal conditions (by estradiol) was observed in cats but not in the dog. The vasa deferentia from castrated or estradiol treated dogs were more responsive to epinephrine than those of normal ones.

Robson (1940) found that the response *in vitro* of the uterus of the bitch and of the cat to oxytocin is reduced by the previous estrogenic treatment of the donors. For the vas deferens of the dog this is not the case. Its responsiveness to pituitrin and to pitocin, in spite of a few tests performed with the latter, was better in estradiol treated animals. The same was previously found in cats (Martins & Valle, 1940).

Vasa deferentia of dogs do not react to histamine and barium unless they come from castrates or estradiol treated castrates. The reaction is predominantly tonic in the former and tonic and rhythmic in the latter group. These drugs, therefore, could be taken as the best pharmacological reagents of the hormonal status of the dog.

SUMMARY AND CONCLUSIONS

The contractility and the pharmacological excitability in vitro of the vas deferens of the dog were studied. The organ was taken from 36 mature, 7 normal and 29 castrated donors. The castrated group was untreated or received testosterone propionate or estradiol benzoate. Two or three vasa deferentia from different dogs were immersed in the same bath of oxygenated Locke's solution at 38–39° C., and the longitudinal contractions simultaneously recorded.

Automatic movements were exhibited only by organs from castrates or from estradiol treated castrates. The vasa deferentia obtained from the estradiol treated castrates were the most responsive to the different drugs assayed. Next came the organs of the untreated castrates, then those of the testosterone treated castrates and, finally, as the least responsive, those of normal donors. Androgens, therefore, exert an inhibitory action and estrogens an opposite one upon the motility of the genital musculature of the dog. The estrogenic treatment sensitizes the vas deferens to the stimulating action of several drugs including epinephrine and oxytocin. Histamine and barium chloride may be regarded as the best reagents for testing the hormonal conditions of the donors; both substances are effective only upon ducts from castrates or estradiol treated castrates.

From these studies we conclude that in the dog, the motility of the vas deferens is under the control of the gonadal hormones.

ACKNOWLEDGMENT

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MOTILITY AND PHARMACOLOGICAL REACTIVITY OF GENITALS OF VITAMIN E DEFICIENT RATS

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As EARLY as 48 hours after castration the vas deferens and the seminal vesicles of rats exhibit spontaneous movements "in vitro" and a pronounced pharmacological reactivity towards common drugs chiefly of the vagomimetic group. These movements and reactions are not shown by the same organs extirpated from normal males under similar experimental conditions (Martins and Valle, 1939; Martins, Valle and Porto, 1940). Organs from rats with sub-normal sized testes exhibit some of the characteristics observed in castrates (Martins and Valle, 1939; Valle and Lamar, 1946). Thus the study of male genitals by the Magnus technique affords an index of the endocrine function of the testes.

It is still unsettled whether in vitamin E deficient rats there is an impairment of testicular hormone output (Mason, 1939; Evans et al. 1939). Evidence in support of the idea that in vitamin E deficient rats there is a testicular hypofunction is the presence in such rats of large basophilic cells of the castrate type in the anterior pituitary gland (van Wagenen, 1925; Nelson, 1933; Mueller and Mueller, 1937 and Koneff, 1939) and an increased content of anterior hypophyseal gonadotropin (Nelson, 1933; Drummond et al. 1939). In such animals, then, the excitability and the pharmacological reactivity of the vas deferens and the seminal vesicles as observed in "in vitro" experiments should be similar to those observed in castrated rats.

MATERIAL AND METHODS

We employed 35 albino male rats. Four normal and 8 castrates were maintained on the diet of our colony (Valle, 1944). Twenty three were placed on the following diet low in vitamin E: starch 50%, casein 16%, lard 13%, dried yeast 15% and salt mixture 3%. Seven animals of the last group received orally 10 to 20 mg. of alpha tocopherol monthly during the period of the experiment. We used litters whose mothers received the vitamin E diet 5 days before parturition and through the lactating period. The females of these litters were saved for control observations. When grown, their vaginal smears were normal but abortion took place 7 to 14 days after conception.

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The effect of the vitamin E deficiency in the males was followed by weekly palpation of the testes. When 11 to 14 months old, the animals were anesthetized with ether and the vas deferentia and seminal vesicles removed, stripped from their connective tissue and immersed in 50–80 ml. of oxygenated Ringer-Locke's solution at 38°–39° C. Three organs coming from donors in different experimental conditions were placed in the same nutritive bath for simultaneous registration of their longitudinal contractions with a load of 2 gm. and a six fold amplification.

After a period of 40 to 60 minutes, sufficient enough to record any spontaneous movements, we observed the action of epinephrine hydrochloride (1:100,000), ephedrine hydrochloride (1:25,000), acetylcholine hydrochloride (1:80,000), pilocarpine hydrochloride (1:25,000) and barium chloride (1:50,000). These drugs were selected as more demonstrative, according to our experience, of the hormonal conditions of the donors.

At the end of the experiment the rats were sacrificed. The testes were removed, weighed, fixed in Stieve's solution and stained with hematoxilin-eosin.

RESULTS

The average testicular weight of rats feed vitamin E low diet plus alpha-tocopherol was 918 mg. and of the vitamin E low group 388 mg. per 100 gm. of body weight. Thus the diet used was effective in

TABLE 1. BODILY AND TESTICULAR WEIGHTS OF DONOR RATS ON A VITAMIN E LOW DIET WITH AND WITHOUT ALPHA-TOCOPHEROL

Vitamin E low diet plus alpha-tocopherol			Vitamin E low diet untreated		
Rat n.	Body weight (gm.)	Testes weight (mg.)	Rat n.	Body weight (gm.)	Testes weight (mg.)
1	370	3150	1	263	1180
3	322	3420	2	275	990
4	326	2460	3	278	760
5	392	3490	4	271	860
6	310	2800	5	286	1190
8	274	3200	7	285	1350
9	354	3000	8	272	1160
M, σ	335 ± 40	3074 ± 360	M, σ	276 ± 20	1070 ± 208

furnishing us with vitamin E deficient rats (Table 1 and Fig. 1). In these animals the morphological macroscopic conditions of the vasa deferentia and of the seminal vesicles were found to show no abnormalities. No significant difference, for example, was found between the weights of dried control seminal vesicles (106 ± 20 mg.) and those of the vitamin E deficient rats (94 ± 23 mg.).

There was no difference in the behavior *in vitro* of the organs coming either from the normal or from the vitamin E deficient groups. But only in one case did the organs of a normal rat show spontaneous contractions and a high excitability to the drugs used. A summary of our results is presented in Table 2. Organs coming from donors reared

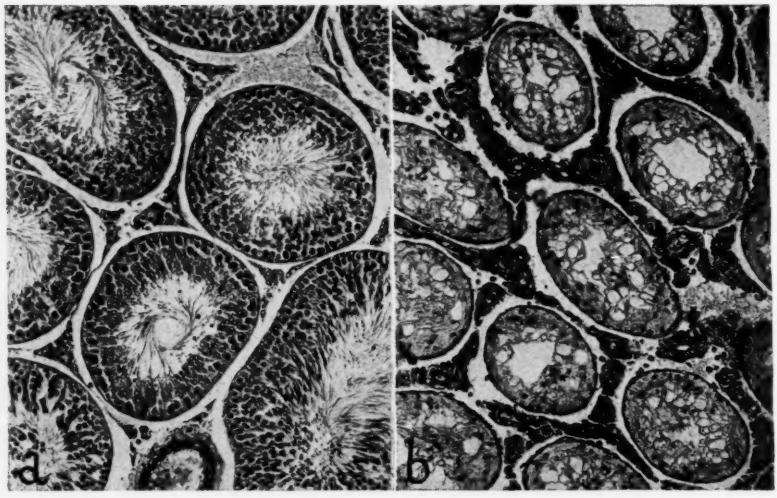

FIG. 1. Sections of rats' testicles (106×). *a*, control, *b*, vitamin E deficient animals.

on the vitamin E low diet with and without alpha-tocopherol did not exhibit automatism or the predominant rhythmic type of response to the pharmacological agents herein studied. The difference of reaction to epinephrine, according to the hormonal conditions of the donors, is exemplified in Fig. 2. Whereas, the deferens coming from a castrated rat exhibited tonic and rhythmic contractions, both organs from rats in vitamin E low diet with and without alpha-tocopherol presented the same feature of response, e.g., a tonic contraction. Inspection of Figs. 3 and 4 affords the same conclusion. Results with pilocarpine

TABLE 2. SUMMARY OF THE EFFECTS OF DRUGS UPON GENITALS OF VITAMIN E DEFICIENT AND CONTROL RATS

Groups	Number of organs studied		Cases showing autom-atism		Total of phar-maco-logical tests	No effect	Oscil-lations	Type of response		
	v.def.	s.ves.	v.def.	s.ves.				Tonic	rhyth-mic	Mixed
Normal donors[1]	10	10	0	1	84	10	16	43	5	10
Vit. E deficient rats	22	22	0	0	165	33	22	83	4	23
Vit. E deficient castrates	6	6	6	4	33	0	1	3	18	11
Castrates	10	11	9	9	71	1	0	17	29	24

[1] This group includes rats in stock diet and in vitamin E diet supplemented with alpha-tocopherol.

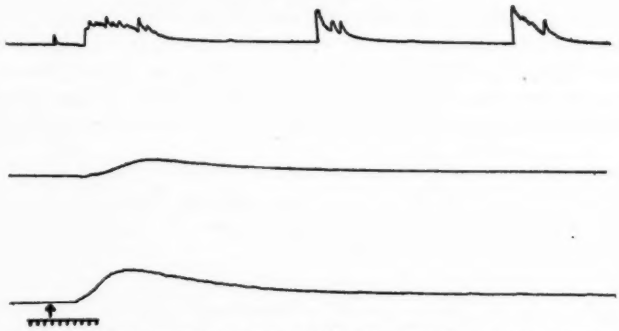

FIG. 2. Three vasa deferentia of rats in the same nutritive bath. *Upper:* from a 5 days' castrated animal; *Middle:* rat n. 1 reared in a vitamin E low diet, treated with α-tocopherol; *Lower:* rat n. 7 reared in a vitamin E low diet. Effects of epinephrine (1:100,000): tonic-rhythmic contractions in the vas deferens from castrate, tonic response in both other organs.

(In all figures time intervals correspond to 5 seconds.)

(Fig. 5) were particularly interesting because this drug provokes response of genitals of male rats when the donors were castrated or castrated and injected with estrogens. Vasa deferentia and seminal vesicles of vitamin E deficient rats, like those of normal controls, did not react to this drug added to the nutritive bath. The study "in vitro" of genitals from 3 vitamin E deficient animals employed the first month after castration showed the same type of behavior exhibited by the castrates from the stock diet.

COMMENTS

New evidence is presented in favor of a sufficient output of the male hormone by the testes of vitamin E deficient rats which is enough

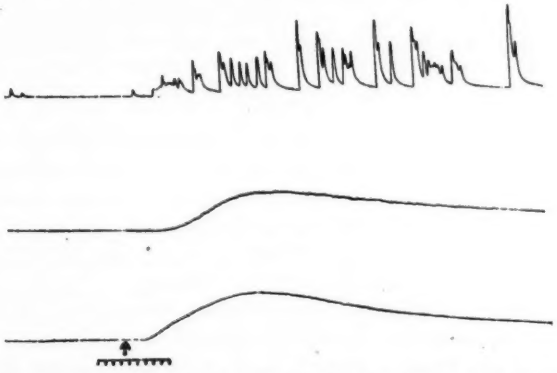

FIG. 3. The same organs as in Fig. 2. Effects of ephedrine (1:25,000). Identical responses of the deferens from control and from vitamin E deficient rat. Tonic-rhythmic contractions only by the castrate.

not only to maintain the average size of genitals but also for their normal pattern of behavior *in vitro*. We know from the literature, however, that the lack of germinal epithelium promotes a histological and functional picture of the anterior lobe of the hypophysis resembling that observed in early castration. Therefore, we are in the

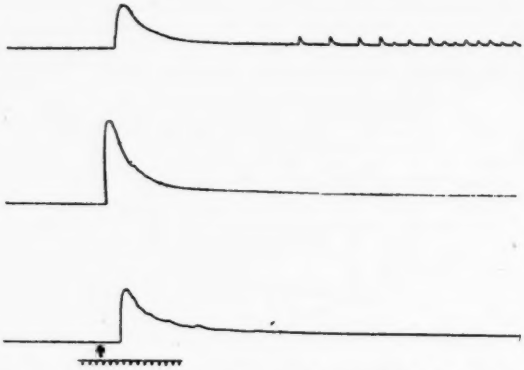

FIG. 4. Seminal vesicles. *Upper:* from a 4 days' castrated rat; *Middle:* rat 1A vitamin E deficient animal; *Lower:* rat n. 3, vitamin E low diet plus α-tocopherol. At the arrow addition to the bath of acetylcholine (1:80,000).

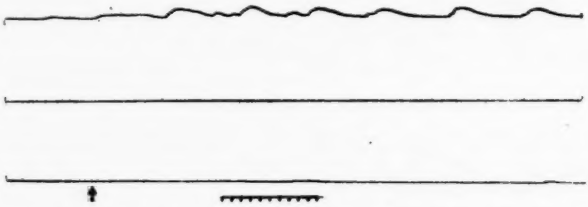

FIG. 5. Vasa deferentia. *Upper:* from a 3 months' castrated rat; *Middle:* rat n. 4, vitamin E low diet plus α-tocopherol; *Lower:* rat n. 8, vitamin E deficient animal. Addition to the bath of pilocarpine (1:25,000). Only the deferens of the castrate reacted to the drug.

same situation as emphasized by Martins (1934) and discussed once more by Moore (1944) for the cryptorchitic rat. The hormonal control on the anterior lobe may be exerted through a second testicular hormone of germinal source or through a higher level of circulating androgens. Clark, Albert and Selye (1942) pointed out that hormonally active steroids can regulate the pituitary gland.

Hessler (1941) studied the motility and the pharmacological reactivity of the uterus of the vitamin E deficient rats. Despite degenerative lesions of the smooth muscle there were no changes of behavior of the uterus *in vitro*, either from the standpoint of spontaneous contractions or from the standpoint of reactions to drugs. Our experi-

ments show that the same is true for the male rats, but in the male the hormonal imbalance in vitamin E deficiency, because of the lack of the germinal epithelium, is a more complicated one than for the non-pregnant female.

SUMMARY

When immersed in Ringer-Locke's solution the vas deferens and the seminal vesicles of castrated rats show spontaneous movements and a high sensitivity to stimulating drugs. The same organs coming from normal or vitamin E deficient animals are quiescent and react poorly to the same pharmacological agents. Thus, as far as the motility of the male smooth musculature is concerned, it seems that there is no decreased output of male hormone by the testes of the vitamin E deficient rat.

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THE RAPID HYPEREMIA PREGNANCY TEST IN EQUIDS. ITS QUANTITATIVE EVALUATION AT DIFFERENT TIME INTERVALS[1]

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JERUSALEM, PALESTINE

IN EARLIER communications we have described a rapid pregnancy test which is suitable for the determination of pregnancy in humans, with an accuracy of 99% within a test period of 8-24 hours (Zondek, Sulman, and Black, 1945; Zondek & Sulman, 1947). The test animal employed in this technic is the infantile female rat, in which species an injection of pregnancy urine elicits the anterior pituitary reaction II (APR II) after a period of 2 hours or more. Marked ovarian hyperemia distinguishes APR in the rat, which differs in kind from APR II in the mouse and rabbit, where it is characterized by follicle hemorrhage (blood points, Zondek and Aschheim, 1926, 1927).

The use of the 24 hr. hyperemia reaction as a routine test of pregnancy in equids has been described (Zondek and Sulman, 1945). It was important to determine whether pregnant mare's blood gonadotropin (PMBG) is similar to human pregnancy urine gonadotropin (PU) in its ability to evoke the hyperemia reaction within a few hours, when minimal quantities of serum are injected. Clarification of this point was of particular interest since the gonadotropin of pregnant mare's blood consists largely of follicle stimulating hormone (FSH), whereas human pregnancy urine contains chiefly luteinizing hormone (LH). It has been found that the hyperemia test is highly sensitive to PU, but much less sensitive to PMBG. One hyperemia unit[2] (APR II) of PMBG is equal on the average to 10 estrus units[3] (APR I), or to about 3 luteinization units[4] (APR III)[5]. The hyperemia test for PMBG therefore requires the use of 10 times as much hormone as the original test for pregnancy in equids (Zondek, 1930 and

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[1] This investigation was aided by a grant from the Rockefeller Foundation.

[2] Hyperemia unit (HU): the minimal quantity of gonadotropin eliciting the hyperemia effect in the ovary of an infantile female rat (cf. later).

[3] Estrus unit (RU): the minimal quantity of gonadotropin eliciting vaginal estrus in an infantile female rat (cf. later).

[4] Luteinization unit (LU): the minimal quantity of gonadotropin eliciting corpus luteum formation in the ovary of the infantile female rat.

[5] The values given represent averages; e.g. the HU value fluctuates between 5 and 20 for different preparations, and this variation may possibly be caused by differences in the relative amounts of FSH and LH.

1935). The rapid test has the advantage however, of giving clear-cut and accurate results in 99% of the cases as early as 12–24 hours after injection.

A positive reaction may be observed in the hyperemia test by the 4th to the 6th hour after the injection of equine gonadotropin; but the reaction is positive at this time in only 33–50% of the animals injected. At this time (4 to 6 hours), therefore, a positive reaction is significant, but a negative result is meaningless. Moreover, the faint pink color of the stimulated ovary at 4 to 6 hours is less easy to recognize than the dark red color observed after 12 to 24 hours. At 4 to 6 hours an additional indicator of a positive reaction is swelling of the ovaries, but since this criterion is dependent on a subjective judgment, it is not considered by us to be satisfactory. For these reasons we give preference to the 12 to 24 hours pregnancy test.

Experiments with PU have shown that the minimum amount of this hormone which suffices to elicit a positive hyperemia reaction is subject to marked fluctuations depending on the time elapsing between the injection and the observation. The time-sensitivity curve of the rat ovary to PU has a parabolic form. The point of highest sensitivity to PU is reached at 10 hours after the injection, at which time the hyperemia unit is superior in sensitivity to all other rodent units. N.B.: one hyperemia unit (10 hours test) = 0.5 estrus unit. But prior to and after 10 hours a positive hyperemia reaction can be elicited only by a considerably larger amount of PU (Zondek and Sulman, 1947). Consequently, it appeared to be of interest to determine whether a similar time relation governs hyperemia as elicited by PMBG.

EXPERIMENTAL

Pregnant mare's blood gonadotropin (PMBG) was used in the following forms: 1) a purified preparation[6] containing 1 IU per 0.1 mg., 2) a sample of the international standard (1 IU = 0.25 mg.), 3) crude acetone-dry powder containing 1 IU per 4 mg. The latter was suspended before use in physiological saline solution and cleared by centrifugation and filtration before injection. Our experiments involved a total of 800 infantile female rats. Fifty animals were examined at each of a series of intervals (4, 6, 8, 10, 11, 12, 18, 20, 24 and 30 hours) to determine the effective unit. In control experiments the estrus and luteinizing unit contents of the preparations were each determined on groups of 10 infantile female rats.

Determination of the hyperemia unit (HU): One HU is the smallest amount of PMBG which elicits a red ovary reaction in 4 to 30 hours after injection. The ovary reaction is considered to be positive if the color resembles that of the spleen, kidney or liver of the test animal. The reaction can be read with certainty 6 to 24 hours after the injection, but is more difficult to interpret at 4 or 30 hours, when the color is not red but pink, at which time a positive reaction can be recognized only by an experienced observer.

[6] We are very much indebted to Dr. G. F. Cartland of Upjohn Co., Kalamazoo, Mich., for a supply of Gonadogen.

In these cases the additional criterion of swelling of the ovary can be applied, but this has the disadvantage of depending on a subjective judgment. One HU was defined as the smallest amount of hormone which elicits a positive reaction in all 8 ovaries (100%) of 4 injected animals.

Determination of estrus unit (RU): One RU is the smallest amount of gonadotropic hormone which produces keratinization (positive vaginal smear) within 72, 84 or 96 hours after injection. The unit is defined as the smallest amount of hormone which elicits a positive reaction in 50% of the animals injected. RU is converted into IU by comparison with a standard preparation.

Results: The findings may be summarized as follows: In the interval of 11 to 24 hours after injection the hyperemia unit of PMBG

TABLE 1. RELATIONSHIP BETWEEN THE PMBG HYPEREMIA UNIT (HU), ESTRUS UNIT (RU) AND INTERNATIONAL UNIT (IU) IN THE INFANTILE FEMALE RAT AT DIFFERENT TIME INTERVALS AFTER INJECTION

Time	4 h	6 h	8 h	10 h	11 h	12 h	18 h	20 h	24 h	30 h
HU	1	1	1	1	1	1	1	1	1	1
RU	100	50	15	15	10	10	10	10	10	20
IU	20	10	3	3	2	2	2	2	2	4

remains constant (1 HU = 2 IU = 10 RU). At shorter or larger intervals a considerably larger amount of PMBG must be given to elicit a positive response (see table 1). When the reaction is read at 4 hours, 20 IU are necessary for a positive response; whereas at 6 hours 10 IU and at 8 and 10 hours 3 IU suffice to elicit a positive response. After 30 hours the sensitivity of the hyperemia test is lower than at the optimal interval of 11 to 24 hours.

DISCUSSION

The results here reported show that the hyperemia response of infantile female rats to PMBG shows precise quantitative relations. The hyperemia unit for PMBG remains constant during the interval of 11 to 24 hours after injection. Our experiments further demonstrate that the hyperemia test can be used as a reliable indicator of pregnancy in equids, provided that the reading is made 11 to 24 hours after injection. The hyperemia test is generally only 1/10 as sensitive as the original four day AZT, based on vaginal estrus and corpus luteum formation, but is consistently positive when 4 cc. of serum are administered, corresponding to a titer of 250 HU = 500 IU of PMBG per liter. Since the 4 day test is 10 times as sensitive (a positive reaction to the injection of 4 cc. corresponding to 250 RU = 50 IU of PMBG per liter of serum), the rapid reaction observed after 11 to 24 hours is best carried out in conjunction with a 4 day test. This point has already been emphasized (Zondek and Sulman, 1945). The rapid test has the advantage that it permits accurate determination of the peak period on the very same day in pregnant mares which are to be bled.

Although it is adequate in principle to base the diagnosis of pregnancy in the mare on the demonstration of the presence of 250 HU = 500 IU of PMBG in the serum, it is advantageous to combine the rapid test with a 4-day test. The latter, being 10 times as sensitive serves to detect cases in which the increase in hormone production is minimal. Cases of this kind occur in the pregnant mare rather often, since in this species gonadotropin does not persist in the serum throughout the entire term of pregnancy and occurs in appreciable quantities only during the interval of 42 to 120 days after impregnation. We have encountered cases in which the hyperemia test was negative shortly before the 42nd day and after the 120th day of the pregnancy. In all these cases pregnancy could be diagnosed by the 4-day test on the basis of examination of the vaginal smear, luteinization being absent. These diagnoses were confirmed by the clinical results. The breeders subsequently suggested that they may have reported erroneous serving times.

The hyperemia reactions evoked by PMBG and PU, respectively, differ in two important respects (Zondek, Sulman and Black, 1945; Zondek and Sulman, 1947):

a) The hyperemia test is more sensitive to PU than to PMBG. One half estrus unit (RU) of PU suffices to elicit a positive hyperemic response, whereas 5 to 20 (with an average of 10) estrus units (RU) of PMBG are required to produce the same effect. The conclusion is therefore suggested that the LH (prolan B) in chorionic gonadotropin is largely responsible for the hyperemia reaction (APR II), which this gonadotropin elicits in the rat ovary; and that FSH, which is present chiefly in PMBG, is relatively inert in this respect. Experiments to test this hypothesis are in progress.

b) The hyperemia test of PU is most sensitive 10 hours after the injection of the hormone. At intervals shorter than 6 hours the test is relatively insensitive, but after this time a gradual progressive increase in sensitivity is observed (Zondek and Sulman, 1947). The hyperemia test of PMBG, on the other hand, is characterized by a constant sensitivity over an extended period of time. At intervals below 6 hours the test is rather insensitive; by 11 hours the maximum of sensitivity has been attained and it is retained from 11 to 24 hours, whereas in the case of chorionic gonadotropin the sensitivity decreases steadily during the same interval.

Furthermore, it is noteworthy that both in the case of PU and PMBG a very large dose of hormone is necessary to elicit the hyperemia reaction before 6 hours.

SUMMARY

The injection of PMBG elicits the anterior pituitary reaction II (APR II = hyperemia reaction) in the rat ovary within 4 to 30 hours.

A positive hyperemia reaction is elicited by 20 IU in 4 hours, by

10 IU in 6 hours, by 3 IU in 8 and 10 hours and by 2 IU in 11 to 24 hours. After more than 30 hours the sensitivity of the hyperemia test gradually decreases, only larger doses of PMBG (4 IU) eliciting a positive reaction.

The results lead to the conclusion that a 4 to 6 hour test is ill-suited to the determination of pregnancy in mares. The 8 to 10 hour test is decidedly more sensitive, and the 11 to 24 hour test is the most reliable of all.

The estrus unit of PMBG is equal to about 0.1 hyperemia units. Since it is difficult to administer more than 4 cc. of mare serum to an infantile rat, the hyperemia test is suitable for pregnancy diagnosis only if the PMBG content of the serum is 250 hyperemia units = 500 IU per liter or more. The original AZT detects pregnancy in the mare by the injection of 4 cc. of serum, even if only 1/10 as much PMBG (250 RU = 50 IU) is present in the serum.

In principle it is adequate to base the diagnosis of pregnancy in the mare on the demonstration of the presence of 250 hyperemia units = 500 IU of PMBG in the serum (11 to 24 hour hyperemia test). Since, however, the gonadotropin level of the blood in the pregnant mare is variable, and is high only during the pregnancy interval of 42 to 120 days, it is suggested that diagnosis of pregnancy in equids should be made on the basis of both the 4-day AZT and the 11 to 24 hour hyperemia test.

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In the last-named reference the reader will find references to the Salmon-Geist-Salmon-Frank 6-hour test for human pregnancy, the forerunner of the test described in this paper.

STUDIES ON ENDOMETRIAL ALKALINE PHOSPHATASE DURING THE HUMAN MENSTRUAL CYCLE AND IN THE HORMONE-TREATED MONKEY[1]

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THE PRESENCE of alkaline phosphatase has been demonstrated histochemically in the uterus by several previous authors (Gomori, 1941; Kabat and Furth, 1941; and Wislocki and Dempsey, 1945). It was found that the enzyme might be present in any of the component tissues of the uterus, the localization varying with the stage of the reproductive cycle and the species in question. More recently, experimental studies in the mouse (Atkinson and Elftman, 1946 and 1947) have shown that treatment with estrogen causes an increase in uterine phosphatase. Progesterone and androgen do not have this effect.

The present studies were undertaken first to determine whether endometrial alkaline phosphatase responded to hormone treatment in the monkey as in the mouse, and secondly to determine whether there are cyclic variations in the phosphatase content of human endometrium during the menstrual cycle.

MATERIALS AND METHODS

In order to ascertain the effect of estrogen and progesterone on the amount and distribution of alkaline phosphatase in the uterus, five ovariectomized rhesus monkeys were studied. These animals had been used in previous experiments but had received no endocrine treatment for at least one year prior to the beginning of the present investigation. The animals were primed with daily intramuscular injections of estradiol benzoate for nine days and the subsequent appearance of uterine bleeding was noted. Three to fourteen days after the cessation of bleeding daily injections of 0.05 mg. of estradiol dipropionate were begun. Two monkeys were treated for ten days and a third for fourteen days. All three animals were sacrificed on the day following the final injection. In the remaining two monkeys the estrogen treatment was stopped after ten days and the animals were then given 2 mgs. of progesterone daily for twelve days. These monkeys were also sacrificed on the day following the final injection. Crystalline hormones were used in these experiments, the daily dose being dissolved in 0.1 to 1.0 cc. of sesame oil.[2]

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[2] The progesterone used in this work was supplied through the courtesy of the

The human material included in this study consists of eighteen endometrial biopsy specimens obtained at various stages of the menstrual cycle. Each specimen was taken from a different woman whose menstrual history was known.[3]

Endometrial specimens from both the women and the monkeys were fixed immediately in Bouin's fluid and in cold (5° to 10° C.) 80% ethyl alcohol. After dehydrating and imbedding in paraffin, sections were cut five to seven microns in thickness. The tissue fixed in Bouin's fluid was stained with Ehrlich's hematoxylin and eosin. The sections of tissue fixed in alcohol were incubated in a sodium glycerophosphate substrate according to the technique of Gomori (1941) for the demonstration of alkaline phosphatase. A slight modification was introduced through the addition of 0.01 M magnesium sulfate to the substrate to increase the phosphatase activity. The sites of enzyme activity in the tissue sections are marked by the deposition of cobalt sulfide in fine black granules. No counterstains were used in order to avoid obscuring these deposits. The sections were mounted in clarite in the usual manner.

OBSERVATIONS

1. ENDOMETRIAL PHOSPHATASE IN THE MONKEY

In both the estrogen and progesterone-treated animals the stromal nuclei contain but slight amounts of alkaline phosphatase and the

TABLE 1. AMOUNT AND DISTRIBUTION OF CYTOPLASMIC ALKALINE PHOSPHATASE IN HUMAN ENDOMETRIUM DURING THE MENSTRUAL CYCLE

Day of cycle	Histological diagnosis	Stroma	Surface epith.	Glandular epith.
8/26	Proliferative	0	+	±
8/	Proliferative	0	++	++
14/	Proliferative	0	+++	++
18/28	Proliferative	0	++	++
29/39	Early Secretory	0	++	+
25/31	Early Secretory	0	*	+
29/33	Early Secretory	0	+	++
22/25	Late Secretory	0	±	±
24/	Late Secretory	0	0	0
26/30	Late Secretory	0	*	0
26/27	Late Secretory	0	0	0
26/27	Premenstrual	0	0	0
27/28	Premenstrual	0	0	0
27/29	Premenstrual	0	0	0
25/25	Premenstrual	0	0	0
24/25	Anovulatory Menstrual	0	0	0
24/24	Anovulatory Menstrual	0	0	0
26/26	Anovulatory Menstrual	0	*	0

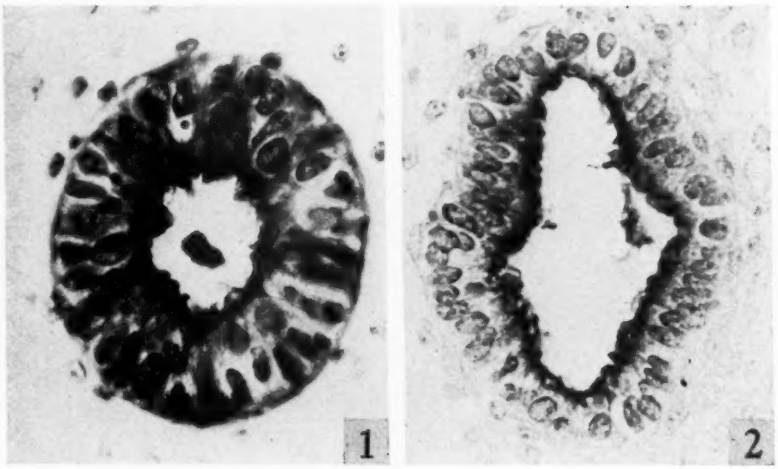
* No surface epithelium present in specimen.

Warren-Teed Products Company, Columbus, Ohio. The estradiol benzoate was made available by CIBA Pharmaceutical Products, Inc., Summit, N. J.

[3] The authors wish to express their appreciation to Dr. C. L. Buxton of Sloane Hospital for his cooperation in providing the endometrial biopsy specimens used in the present study.

cytoplasm of the stromal cells is devoid of enzyme activity. The activity in the endometrial vessels is variable. Many of the arterioles and capillaries show maximal reactions, whereas no response has been noted in either the venous sinuses or venules. It is interesting to note that, in contrast to the mouse, the myometrium of the monkey contains little or no phosphatase.

The surface and glandular epithelium in the animals treated with



Photomicrographs (×500) of the cross sections of glands lying in the endometrium functionalis. The photographs were made under comparable conditions so that the intensity of alkaline phosphatase activity can be estimated from the density of silver deposition.

FIG. 1. Estrogen-treated monkey: the glands are straight, with narrow lumens. The epithelium is high columnar, the nuclei are oval and stratified. The alkaline phosphatase is concentrated in the nuclei and in the apical cytoplasm.

FIG. 2. Progesterone-treated monkey: the glands are coiled, with a moderate degree of convolution and plication. The epithelium is lower, the nuclei are rounded and situated basally. The alkaline phosphatase is greatly reduced except at the free borders of the cells.

estrogen alone exhibits large amounts of alkaline phosphatase. In the basalis of the endometrium the terminal portions of the glands have the enzyme distributed throughout the cytoplasm of the cells. In the portions of the glands lying in the functionalis the phosphatase activity appears to be concentrated in the cytoplasm apical to the epithelial nuclei (Fig. 1). The reaction in the nuclei is pronounced and is uniform throughout the entire gland. In the monkeys treated with progesterone, however, there is a marked reduction in the enzyme activity in the surface epithelium and in the glands of the functionalis (Fig. 2). In the basalis the phosphatase activity is equal to that in the estrogen-treated animals. The reaction of the epithelial nuclei also remains unchanged.

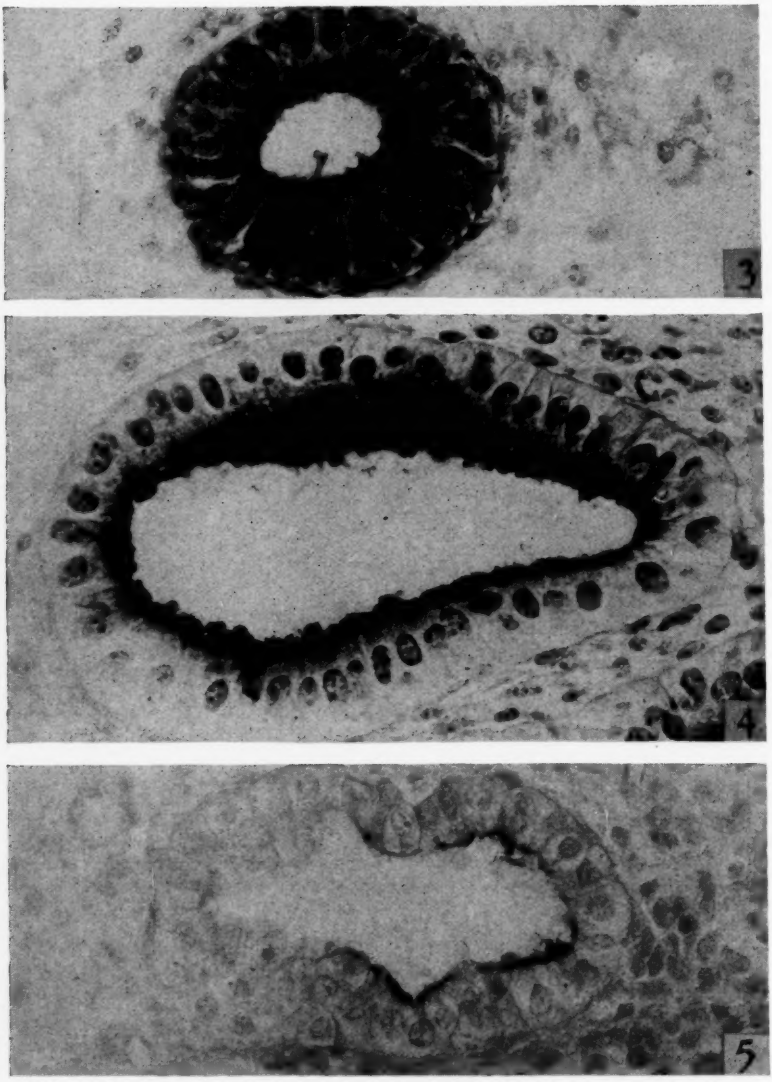


FIG. 3. Typical gland from a human endometrial biopsy taken on the 18th day of a 28 day cycle. The glands are straight, with narrow lumens. The epithelial cells are columnar, the nuclei oval and stratified. The alkaline phosphatase activity of the nuclei is marked, the enzyme activity of the cytoplasm is greatest in the apical portions of the cells.

FIG. 4. Secretory gland from the endometrium of a woman biopsied on the 29th day of a 33 day cycle. The glands are coiled, with some infolding. The nuclei are rounded

2. ENDOMETRIAL PHOSPHATASE IN THE HUMAN

Since this material was obtained by the biopsy method, the endometrial basalis has not been examined. The following descriptions relate to the components of the functionalis only. As in the hormone-treated monkey, the stromal nuclei show but slight phosphatase activity and the cytoplasm of these cells is entirely negative. The enzyme distribution in the arterioles and capillaries is also variable. The concentration of phosphatase in the stroma and endometrial vessels is relatively constant, there being little variation in enzyme activity from one stage of the menstrual cycle to the next.

The amount of alkaline phosphatase in the uterine glands and surface epithelium, on the other hand, varies considerably throughout the menstrual cycle. During the phase of endometrial proliferation moderate to dense deposits of cobalt sulfide are seen in the cells of both the surface and glandular epithelium and in the lumina of the glands themselves (Fig. 3). The deposition is most dense at the free surface of the cells and in the cytoplasm apical to the nuclei. The epithelial nuclei exhibit considerable enzyme activity as compared with the nuclei of the stromal cells.

During the early secretory phase (16th to 19th days) the distribution of phosphatase in the endometrium is similar to that described in the proliferative phase. There is a decrease in the enzyme activity in both the surface and glandular epithelium (Fig. 4). The secretion contained in the lumina of the glands continues to show marked phosphatase activity. However, it is difficult to estimate accurately any change in the amount of enzyme activity in the glandular secretions since much of this material is lost during the manipulation of the tissue sections.

There is a sharp reduction of enzyme activity in the surface and glandular epithelium by the late secretory phase (24th to 25th days) (Fig. 5). At this time and during the premenstrual phase (26th to 27th days) there is little or no phosphatase activity in the endometrium other than in the endothelium of the arterioles. It is not uncommon to find a narrow line of cobalt sulfide deposited along the free borders of the surface and glandular epithelium. However, this is probably the result of phase-boundary deposition of the reagents rather than an indication of enzyme activity. The distribution of the enzyme in three anovulatory menstrual specimens was the same as that seen in the premenstrual secretory endometrium.

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and basally located. There is a reduction of alkaline phosphatase in the nuclei and in the cytoplasm except at the apices of the cells.

FIG. 5. Gland from a premenstrual endometrial biopsy obtained from a woman on the 27th day of a 29 day cycle. The glands are highly convoluted. The epithelium is columnar with apical blebs. The nuclei and cytoplasm of the cells are devoid of alkaline phosphatase activity. Occasional traces of the enzyme may be seen at the free border of the cells.

DISCUSSION

The present studies have indicated clearly that there are considerable quantities of alkaline phosphatase in the surface and glandular epithelium of the human endometrium during the proliferative phase of the menstrual cycle and in the ovariectomized monkey following estrogen treatment. These findings are in agreement with previous work (Atkinson and Elftman 1946) which demonstrated that estrogen mobilized alkaline phosphatase in the uterus of the mouse. The presence of the enzyme throughout the entire epithelial cell in the monkey is probably the result of the experimental procedure rather than a significant species difference from the human. This is suggested by the observation in the mouse that after short periods of estrogen treatment the enzyme is localized in the apical portions of the epithelial cells, whereas the enzyme becomes distributed throughout the entire cell after more prolonged treatment (Atkinson and Elftman 1947). It has been shown also in the present work that there is a marked reduction in epithelial phosphatase during the secretory phase of the menstrual cycle of the human and in the functionalis of the endometrium of the progesterone-treated monkey. The endometrial glands of the basalis in the monkey do not show any appreciable reduction in enzyme activity. In this connection, however, it should be remembered that only the more superficial endometrium is obtained in the human biopsy specimens. It is also significant to note that only the portions of the glands in the endometrial functionalis exhibit morphological alterations during the menstrual cycle and respond readily to hormone stimulation.

The function of alkaline phosphatase in the uterus remains obscure. Cytochemical studies alone serve only to localize the enzyme and to give a rough estimate of its concentration. However, by comparing the biochemical potentialities of phosphatase with the physiological processes known to occur in the tissues under consideration, possible functions can be suggested tentatively.

The biochemical and physiological functions of the phosphatases have been discussed recently in two excellent reviews (Dempsey and Wislocki, 1946; Moog, 1946). In brief, the fundamental biochemical significance of the phosphatases is their ability to catalyze the hydrolysis of phosphoric esters. The synthesis of these esters is accomplished by the adenosine polyphosphate system with its separate group of enzymes. Among the important physiological processes involving the dephosphorylation of organic compounds may be included the intermediate metabolism of the nucleoproteins, neutral fats and glycogen. Phosphatase may function directly in the above processes or may play an indirect role by supplying necessary phosphate ions from outside sources.

Estrogen has innumerable effects on uterine physiology, one of the

most marked being the stimulation of growth, particularly in the endometrial glands. This is most pronounced during the proliferative phase of the menstrual cycle. In view of the fact that growth processes are generally accompanied by an increase in the rate of protein synthesis, the appearance of alkaline phosphatase at this time is highly suggestive of a functional relationship between the two estrogen-induced phenomena. The simultaneous appearance of phosphatase and glycogen and disappearance of lipids in the endometrial glands also may indicate an interrelationship between these substances. Further data along these lines are needed before final solutions to these problems are possible.

SUMMARY

The distribution of alkaline phosphatase has been studied histochemically in the endometrium of the hormone-treated ovariectomized monkey and in endometrial biopsies obtained at various stages throughout the human menstrual cycle. In the monkey treated with estrogen alone the enzyme is found chiefly in the endometrial glands and in the surface epithelium. When estrogen is followed by progesterone there is a considerable reduction of phosphatase in the surface epithelium and in the portions of the glands lying in the functionalis. Marked variation in the enzyme content of these tissues is seen also during the menstrual cycle in women. The alkaline phosphatase activity, high during the proliferative phase, is reduced during the secretory phase and disappears several days before the onset of menstruation.

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THE THYROXINE-LIKE ACTION OF ELEMENTAL IODINE IN THE RAT AND CHICK[1]

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THE *in vitro* formation of iodinated proteins, which have thyroidal activity when administered to birds and mammals, has been the subject of numerous investigations (Abelin and Florin, 1933; Abelin, 1934; Abelin, 1936; Abelin and Neftel, 1938; Reinecke, Williamson and Turner, 1942; Salter, 1944). Iodination of albumin was shown to yield 3-5 diiodotyrosine by Oswald in 1911. This was confirmed by Ludwig and von Mutzenbecker in 1939, who also succeeded in isolating thyroxine from iodinated casein, albumin, globulin, fibroin and edestin. The isolation of thyroxine from iodinated proteins was confirmed by Harington and Pitt-Rivers in 1939, and Reinecke and Turner in 1943. Thyroxine was also recovered by the *in vitro* incubation of diiodotyrosine in sodium hydroxide by von Mutzenbecker in 1939, and by Block in 1940.

The *in vivo* administration of elemental iodine (I_2)[2] via parenteral routes induces metamorphosis in the axolotl (Hirschler, 1922; Abelin, 1927; Blacher and Belkin, 1927; Ingram, 1929), even after thyroidectomy or hypophysectomy (Blacher and Belkin, 1927; Ingram, 1929). The addition of elemental iodine to the water in which the normal axolotl is present does not do this (Abelin, 1927).

The parenteral administration of elemental iodine (Hirschler, 1922), or the oral administration of elemental iodine or iodide ($I-$) in food, to normal anuran larvae (Swingle, 1919) accelerates metamorphosis. The addition of iodide to the water in which tadpoles are present is claimed to accelerate metamorphosis (Abderhalden and Hartman, 1927). This is denied by Morse (1914) who claims that neither elemental iodine nor iodide exerts any stimulating effect when added to the water. Iodinated serum albumin is effective, however. As is well known, diiodotyramine, diiodotyrosine, thyroxine, acetyl-thy-

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[1] Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation.

[2] To avoid confusion, the following terminology is used in this paper: elemental iodine (I_2), iodide (I^-), this being in the form of sodium iodide (NaI), and iodine. This latter term, iodine, is used in a collective or generic sense, and does not here indicate whether this halogen is elemental or an iodide.

roxine and thyroid gland powder are also effective in accelerating anuran metamorphosis.

It seemed of interest to determine if the *in vivo* administration of iodine to birds and mammals would produce a response similar to that elicited by thyroid gland powder and thyroxine. In order to determine this, tests were made of the ability of iodine to simulate four known actions of thyroid gland powder and thyroxine. The actions tested were: 1) The ability to restore and maintain growth after thyroidectomy. Thyroid powder fed to rats thyroidectomized at forty days or at birth causes maintenance of growth (Smith, Greenwood and Foster, 1927; Salmon, 1938). Thyroxine (Evans, Simpson and Pencharz, 1939; Rowlands, 1942) and iodinated proteins (Rowlands, 1945) also restore growth in young thyroidectomized rats or goats (Reinecke and Turner, 1941). 2) The ability to restore the adrenal gland weight of thyroidectomized rats to normal. The administration of thyroid powder and thyroxine does this (Smith, Greenwood and Foster, 1927; Rowlands, 1942). 3) The ability, in normal rats, to cause involution of the thyroid gland epithelium and to decrease the weight of the gland. Thyroid powder, when fed, has this action (Herring, 1917; Cameron and Carmichael, 1920). Thyroxine has similar activity (Uotila, 1940; Cortell and Rawson, 1944). 4) The ability to prevent the hypertrophy of the thyroid gland epithelium, and to prevent the increase in weight of the thyroid gland due to the administration of thiouracil or sulfadiazine to normal rats. Thyroxine has this action in normal rats fed thiouracil (Dempsey and Astwood, 1943; Astwood and Bissell, 1944; Reinecke, Mixner and Turner, 1945) or sulfaguanidine (MacKenzie and MacKenzie, 1943; Astwood, Sullivan, Bissell, and Tyslowitz, 1943) or sulfadiazine (Astwood and Bissell, 1944). This action is not prevented by diiodotyrosine (Dempsey and Astwood, 1943), and only slightly inhibited by iodide in the case of animals fed 0.1% thiouracil (Astwood, 1945). The normal iodine content of the diet does inhibit the thyroid-weight increase when low dosages of thiouracil (0.01%) are fed to rats (Astwood, 1945). Iodide fails to inhibit the goiterogenic effect of sulfaguanidine (MacKenzie and MacKenzie, 1943), and only slightly inhibits the thyroid hyperplasia of thiourea fed rats (Astwood, et al., 1943). Thyroxine inhibits the goiterogenic action of thiouracil fed to chicks (Mixner, Reinecke and Turner, 1944).

Experiments were designed to test the action of iodine in the form of sodium iodide and of elemental iodine in regard to these four responses to thyroid material. Further studies were made to test whether the site of administration, either oral or parenteral, influenced the responses. Thyroxine treated and untreated animals served as controls in most groups.

Administration of thiouracil by the subcutaneous implantation of pellets was carried out in some groups, and was the sole method of

administration of this drug when elemental iodine or iodide was given orally.

MATERIALS AND METHODS

Young female rats of the Long-Evans strain were used. They ranged in age from 25 to 70 days at the start of the experiment.

Thyroidectomy[3] was performed in 85 rats at 39 to 47 days of age. Body length was measured at biweekly intervals after deeply anesthetizing the rats with ether. Only those rats which showed a marked retardation in rate of growth in length and of weight increase, and which had no remnant of thyroid tissue present in serial sections of the trachea and surrounding structures from a level above the larynx to the entrance of the trachea into the thorax, were used. Only nine rats fulfilled these requirements. Three of these remained untreated and served as thyroidectomized controls. Two were treated with daily subcutaneous injections of 8.9 mg. of iodide in 0.2 cc. of water. The injections were given during a 22 day period starting at 83 and 84 days after thyroidectomy, and later during a period of 25 days, starting at 160 and 161 days after thyroidectomy. Two other thyroidectomized rats were treated with daily subcutaneous injections of 5.6 mg. of iodide plus 3.3 mg. of elemental iodine in 0.2 cc. of water, for a period of 22 days, starting at 84 and 86 days respectively after thyroidectomy, and later for a period of 25 days, starting at 161 and 163 days after thyroidectomy. The dosage of iodine administered was equal to the total dosage used in the preceding group. Another two were similarly treated for 28 days, starting at 97 and 98 days after thyroidectomy, and later for 43 days, starting at 182 and 183 days after thyroidectomy. At autopsy, the tibias of these rats were fixed in Bouin's fluid and decalcified. Parasagittal sections of the head of the tibia were cut at 15 μ and stained with hematoxylin and eosin. The adrenal gland were dissected free and weighed.

Normal rats were treated for from 10 to 42 days by the daily subcutaneous injection of both the iodide and the iodide plus elemental iodine solutions mentioned above. Other rats were force fed daily for a 17-day period, with equal amounts of iodide or iodide plus elemental iodine contained in 4 cc. of water. In order to simulate the necrosis of the skin which occurred when elemental iodine was injected, 0.2 cc. of 95% ethanol was injected subcutaneously each day for 13 days into normal animals. Others were injected with 8.9 mg. of iodide in 0.2 cc. of 95% ethanol. One group was treated daily for 13 days with 7.5 μg. of d,l-thyroxine by subcutaneous injection. Another group remained untreated and served as a control.

Thiouracil[4] was fed to normal rats as 0.1% of the food. It was also administered to other rats by implantation of pellets measuring 9 mm. in diameter and about 3 mm. in height and weighing from 185 mg. to 271 mg. The pellets were sterilized in individual vials by dry heating at 120° C. for two hours, and implanted, aseptically, into the inguinal region. At autopsy, 13 days later the pellets were freed from the surrounding connective tissue capsule, rinsed in tap water, and dried to a constant weight by heating in an air oven at 100° C. The average daily dosage was calculated by dividing the difference

[3] Since the parathyroids are embedded in the thyroid gland, they were also removed at operation.

[4] The thiouracil was supplied through the kindness of the Calco Chemical Division of the American Cyanamid Company.

in pellet weight before implantation and after removal by the number of days of treatment.

Iodide in three dosages was administered concurrently with the thiouracil treatments. One dosage was 8.9 mg. of iodide in 0.2 cc. of water administered by daily subcutaneous injections. Some of the animals treated with this dosage received thiouracil in food, others in pellet form. A similar dosage of iodide was dissolved in 4 cc. of water and administered to another group by daily force feeding. This group received thiouracil by subcutaneous pellet implant only. In order to produce skin necrosis in a group injected with iodide only, 95% ethanol was used as the solvent, and 8.9 mg. of iodide in 0.2 cc. was injected each day. Another group was injected with 0.2 cc. of 95% ethanol alone. A second dosage, administered by daily subcutaneous injection, was 2.25 mg. of iodide in 0.1 cc. of water. A third dosage, administered by daily subcutaneous injection, was 0.225 mg. of iodide in 0.1 cc. of water. The animals treated with the latter two dosages were given thiouracil either orally, in food, or by subcutaneous pellet implants.

Similar experiments using iodide plus elemental iodine were set up with dosages of iodine equal to the iodide dosages administered in the earlier groups, elemental iodine being about one-third of each dosage. Thus, solutions were injected daily containing 5.6 mg. of iodide plus 3.3 mg. of elemental iodine, 1.4 mg. of iodide plus 0.85 mg. of elemental iodine and 0.14 mg. of iodide plus 0.085 mg. of elemental iodine. The largest dosage was given also by the oral route in animals treated with thiouracil pellets. One group received 7.5 μg.[5] of d,l-thyroxine per day by subcutaneous injection, and another group remained untreated except for the administration of thiouracil.

Experiments similar to those with unoperated rats were done on day-old White Leghorn chicks. One group was untreated and served as controls. A second group received thiouracil only, as 0.1% of the diet. A third group, fed with the thiouracil-containing diet, was injected daily with 5.4 mg. of iodide in 0.12 cc. of water. A fourth group, also fed with the thiouracil-containing diet, was injected daily with 3.4 mg. of iodide plus 2.0 mg. of elemental iodine in 0.12 cc. of water. The chicks were kept in a heated brooder. The experiment lasted 10 days.

Sulfadiazine was fed to normal young female rats for 10 days as 1.0% of the diet. One group received sulfadiazine only. Another group, fed the sulfadiazone-containing diet, was injected subcutaneously each day with 8.9 mg. of iodide in 0.2 cc. of water. A third group, also fed the sulfadiazine-containing diet, was injected dialy with 5.6 mg. of iodide plus 3.3 mg. of elemental iodine in 0.2 cc. of water. A fourth group, was fed a regular diet, and served as controls.

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[5] Dempsey and Astwood (1943) found that daily injections of 5.2 μg. of d-l-thyroxine were required to maintain a normal thyroid weight in young male rats fed thiouracil and kept at 25° C. Reinecke, Mixner and Turner (1945) found that 4.8 μg. of d-l-thyroxine per day was needed to prevent the thyroid enlargement when thiouracil was fed. Reinecke and Turner (1945) call attention to the fact that d-l-thyroxine is one-half as active as l-thyroxine in preventing the goiter due to thiouracil administration. They note that the above estimates of effective dosages of thyroxine differ by about 100%.

In this experiment, a dosage of 7.5 μg. of d-l-thyroxine per day was used since it contains an amount of l-thyroxine intermediate between that used by Dempsey and Astwood (1943) and Reinecke, Mixner and Turner (1945).

At autopsy the thyroids were carefully dissected free under a binocular microscope, and weighed to the nearest milligram. They were then fixed in Bouin's fluid, sectioned at 7 μ and stained with hematoxylin and eosin. The cell height of the thyroid epithelium of each animal was determined with an ocular micrometer by measuring a cell of 25 successive follicles from the mid-portion of the gland. The results were faily consistent within each group, though fewer measurements were made than by Rawson and Starr (1938) and Uotila (1940), where a cell from 200 and 100 follicles, respectively, in each animal was measured.

The mean and standard deviation of the weight of the thyroid per 100 gm. of body weight and the cell height of the thyroid epithelium of each group were calculated, using Fisher's (1944) formulae for small groups. The value of P was also calculated so as to compare the means of different groups. Only a P value of less than 0.01 was regarded as evidence of a significant statistical difference.

RESULTS

1. Thyroidectomized Group. Following the ablation of the thyroid the rate of growth of body length was decreased in 9 of the young female rats (Fig. 1). Growth nearly ceased at about 60 days after thyroidectomy. Those which were injected with iodide showed only a very slight resumption of growth. Those, however, which were injected with an equivalent amount of iodine, about two-thirds being

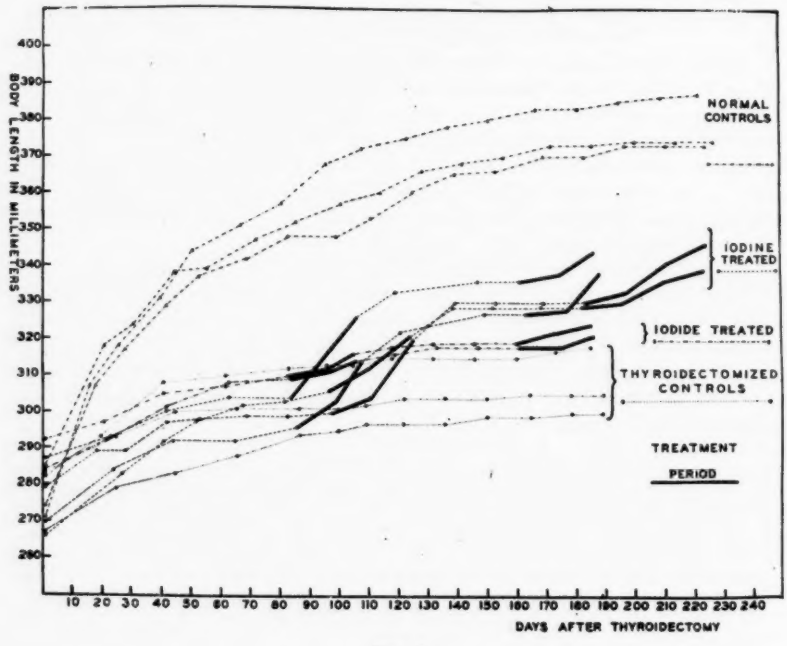

FIG. 1

iodide and one-third elemental iodine, showed a very striking increase in body length. In general, the increase was less rapid during the earlier half of the treatment period than during the latter half. Also, growth continued for about two weeks after treatment had been stopped. After growth ceased, a resumption was again induced by injections of a solution containing elemental iodine, whereas only slight effects were apparent when a solution containing iodide alone was injected.

The histological structure of the head of the tibia varied markedly among the different groups. The untreated thyroidectomized rats had tibias with a thin epiphyseal plate with a narrow zone of cellular and lacunar enlargement. The subjacent marrow cavity rarely contained bony trabeculae (Fig. 2). The normal untreated controls at the age of about 265 days, when growth in length had nearly ceased, had epiphyseal plates which were slightly thinner, and had coarse bony

TABLE 1. ADRENAL WEIGHTS OF THYROIDECTOMIZED (THRYX.) FEMALE RATS TREATED WITH INJECTIONS CONTAINING IODIDE (I^-) OR IODIDE PLUS ELEMENTAL IODINE (I_2)

Type of animal	Treatment	No. of anim.	Adrenal wt., mg.				Adrenal wt., mg./100 gm. body wt.			
			Av.	Range	Ratio of av.		Av.	Range	Ratio of av.	
					Norm. =1.00	ThryX. =1.00			Norm. =1.00	ThyrX. =1.00
Normal Controls	None	5	50.7	36.3–62.4	1.00	2.86	23.7	17.8–28.3	1.00	1.77
ThryX.	None	3	17.7	13.9–21.1	0.35	1.00	13.4	10.2–16.6	0.57	1.00
ThryX.	I^-	2	21.0	20.8–21.2	0.41	1.19	13.1	12.9–13.2	0.55	0.98
ThryX.	I^-&I_2	4	43.5	38.4–50.0	0.86	2.46	24.3	20.1–28.4	1.03	1.82

trabeculae in the subjacent marrow cavity (Fig. 3). Thyroidectomized rats, treated with injections of iodide had a wider zone of cartilage cell enlargement and more fine trabeculae of bone beneath the epiphyseal plate than the untreated thyroidectomized controls (Fig. 4). The thyroidectomized rats injected with iodide plus elemental iodine had epiphyseal plates which were decidedly thicker than those of the other groups. The columns in the zone of cellular and lacunar enlargement were longer and each lacuna appeared larger than in the other groups. Numerous fine trabeculae of bone were present under the cartilage, and these extended for a greater distance from the cartilage than in any of the other groups (Fig. 5). In this group, the sequential changes of osteogenesis were most prominent.

Study of adrenal weights (Table 1) reveals that thyroidectomy resulted in a lowering of adrenal weight to about one-third of normal, or when computed as weight per 100 gm. of body weight, to about two-thirds of normal. Injections of iodide solution failed to alter this weight. However, injections of a solution of iodide plus elemental iodine restored adrenal weight to essentially normal levels.

2. Normal Group. Normal young female rats in our colony have an average thyroid weight of 7.2 mg. per 100 grams of body weight

TABLE 2. THYROID WEIGHT AND THYROID EPITHELIUM CELL HEIGHT OF NORMAL YOUNG FEMALE RATS TREATED WITH IODIDE (I^-) OR IODIDE PLUS ELEMENTAL IODINE (I_2) OR THYROXINE

Treatment				No. of anim.	Thyroid wt., mg./100 gm. body wt.				Cell height—μ			
I^- mg./d.	I_2 mg./d.	Site	Days		Av. $\pm\sigma$	Ratio norm.	P	P_1	Av. $\pm\sigma$	Ratio norm.	P	P_1
None	None			20	7.2 ±1.5	1.00			9.7 ±1.4	1.00		
8.9[1]	None	sub. cut.	13	4	7.5 ±0.2	1.04	0.7		7.1 ±0.2	0.73	<0.01	
8.9	None	sub. cut.	10–42	12	7.1 ±0.9	0.99	0.8		7.6 ±0.9	0.78	<0.01	
8.9	None	oral	17	5					7.4 ±0.1	0.76	<0.01	
5.6	3.3	sub. cut.	10–42	16	5.9 ±0.7	0.82	<0.01	<0.01	4.4 ±0.8	0.45	<0.01	<0.01
5.6	3.3	oral	17	5					7.2 ±0.5	0.74	<0.01	<0.4
Ethanol, 95% 0.2 cc./d.		sub. cut.	13	4	7.7 ±0.7	1.07	0.8		9.8 ±0.0	1.01	0.9	
Thyroxine 7.5 μg./d.		sub. cut.	13	4	4.9 ±0.9	0.68	<0.01	>0.05	5.1 ±0.1	0.53	<0.01	<0.01

[1] =Sodium iodide dissolved in 95% ethanol. Aqueous solution used in other groups.

P = P value of groups compared with normal controls.

P_1 = P value of groups compared with groups similarly treated with iodide only. The thyroxine treated group is compared with the group treated with iodide plus elemental iodine by subcutaneous injection.

TABLE 3. THYROID WEIGHT AND CELL HEIGHT OF THYROID EPITHELIUM OF NORMAL YOUNG FEMALE RATS TREATED WITH THIOURACIL AS 0.1% OF THE DIET OR AS PELLET IMPLANTS, AND ALSO TREATED WITH IODIDE (I^-) OR IODIDE PLUS ELEMENTAL IODINE (I_2) OR THYROXINE FOR 13 DAYS

Treatment			No. of anim.	Thyroid wt., mg./100 gm. body wt.					Cell height—μ				
I^- mg./d.	I_2 mg./d.	Site		Av. $\pm\sigma$	Ratio thio. tr. = 1.00	P	P_1	P_2	Av. $\pm\sigma$	Ratio thio. tr. = 1.00	P	P_1	P_2
None	None		13	23.9 ±2.7	1.00				13.5 ±0.4	1.00			
8.9[1]	None	sub. cut.	5	18.8 ±1.3	0.79	<0.01	>0.9		13.9 ±0.2	1.03	>0.02	<0.01	
8.9	None	sub. cut.	9	18.8 ±1.2	0.79	<0.01			13.3 ±0.4	0.99	0.3		
8.9	None	oral	4	14.5 ±2.1	0.61	<0.01		<0.01	13.0 ±0.5	0.96	0.05		0.3
2.25	None	sub. cut.	5	14.7 ±1.7	0.62	<0.01		<0.01	13.3 ±1.0	0.99	0.5		>0.9
0.225	None	sub. cut.	5	16.8 ±2.3	0.70	<0.01		>0.05	12.8 ±1.0	0.95	0.05		0.2
5.6	3.3	sub. cut.	19	7.1 ±1.1	0.29	<0.01	<0.01		7.5 ±2.1	0.56	<0.01	<0.01	
5.6	3.3	oral	4	15.9 ±1.0	0.67	<0.01	0.3	<0.01	13.6 ±0.3	1.01	0.7	0.9	<0.01
1.4	0.85	sub. cut.	10	14.9 ±2.6	0.62	<0.01	0.9	<0.01	11.1 ±1.5	0.82	<0.01	<0.02	<0.01
0.14	0.085	sub. cut.	7	15.7 ±1.7	0.66	<0.01	0.4	<0.01	10.2 ±2.0	0.76	<0.01	<0.05	<0.01
Ethanol, 95% 0.2 cc./d.		sub. cut.	3	23.0 ±1.6	0.96	0.6			13.7 ±0.1	1.01	0.5		
Thyroxine 7.5 μg./d.		sub. cut.	4	5.8 ±1.3	0.24	<0.01			5.4 ±0.4	0.40	<0.01		

[1] = Sodium iodide dissolved in 95% ethanol. Aqueous solution used in other groups.
P = P value of groups compared with thiouracil treated controls.
P_1 = P value of groups compared with groups treated with equal total iodine dosage in the form of iodide (I^-) only.
P_2 = P value of groups compared with groups receiving the largest amount of a similar type of iodine solution.

(Table 2). No statistically significant alteration in this value was observed in the groups injected with iodide. The group injected with iodide plus elemental iodine, however, had an average thyroid weight significantly lower than normal, and one which approximated that of the thyroxine injected rats (Table 2).

The average cell height of the thyroid epithelium of normal control rats was 9.7μ (Table 2; Fig. 6). In rats injected with iodide, the cell height was 7.6μ (Table 2, Fig. 7). Rats injected with similar total iodine dosage, except that about one-third was in the form of elemental iodine, had an average cell height of 4.4μ (Table 2, Fig. 8), a value significantly lower than that of the group receiving iodide only, and even lower than after thyroxine treatments (Table 2, Figs. 8, 9). In the elemental iodine and thyroxine treated animals, the colloid was more eosinophilic and abundant than in the normal untreated or iodide treated animals.

Oral administration of iodide alone, or iodide plus elemental iodine, caused a reduction of average thyroid cell height (Table 2). The cell height was similar to that resulting from an equivalent total dosage of iodide given subcutaneously (Table 2).

3. Thiouracil Treated Groups. a. Rats. Thiouracil, when administered in pellet form, was absorbed at an average rate of 9.4 mg. per day. When administered in food the average dosage was 13.1 mg. per day. The average thyroid weight per 100 gm. of body weight was 23.9 mg., a weight over three times that in normal animals (Table 3).

Iodide, or iodide plus elemental iodine, via any route and at all dosage levels administered, significantly lowered thyroid weight, and partly, or completely, inhibited the goiterogenic effect of thiouracil. At the lower dosages, there seemed to be no significant proportional relationship between the degree of inhibition of weight increase and the amount or type of iodine administered. Nor did the method of administration influence the response. However, with the largest dosage, there was a much greater inhibition produced by iodide plus elemental iodine than by iodide alone, when equivalent total amounts of iodine were injected. Thus, the average thyroid weight per 100 gm.

PLATE 1. Parasagittal section through the head of the tibias of female rats. ×120.

FIG. 2. Thyroidectomized rat, 229 days old, and 182 days after operation.

FIG. 3. Normal unoperated 265 day old rat.

FIG. 4. Thyroidectomized rat, 228 days old, and 186 days after thyroidectomy. Treated during two periods totalling 47 days, the latter period starting 25 days before autopsy, with daily subcutaneous injections of 8.9 mg. of iodide (I^-).

FIG. 5. Thyroidectomized rat, 229 days old, and 182 days after thyroidectomy. Treated during two periods totalling 47 days, the latter period starting 25 days before autopsy, with daily subcutaneous injections of 5.6 mg. of iodide plus 3.3 mg. of elemental iodine (I_2).

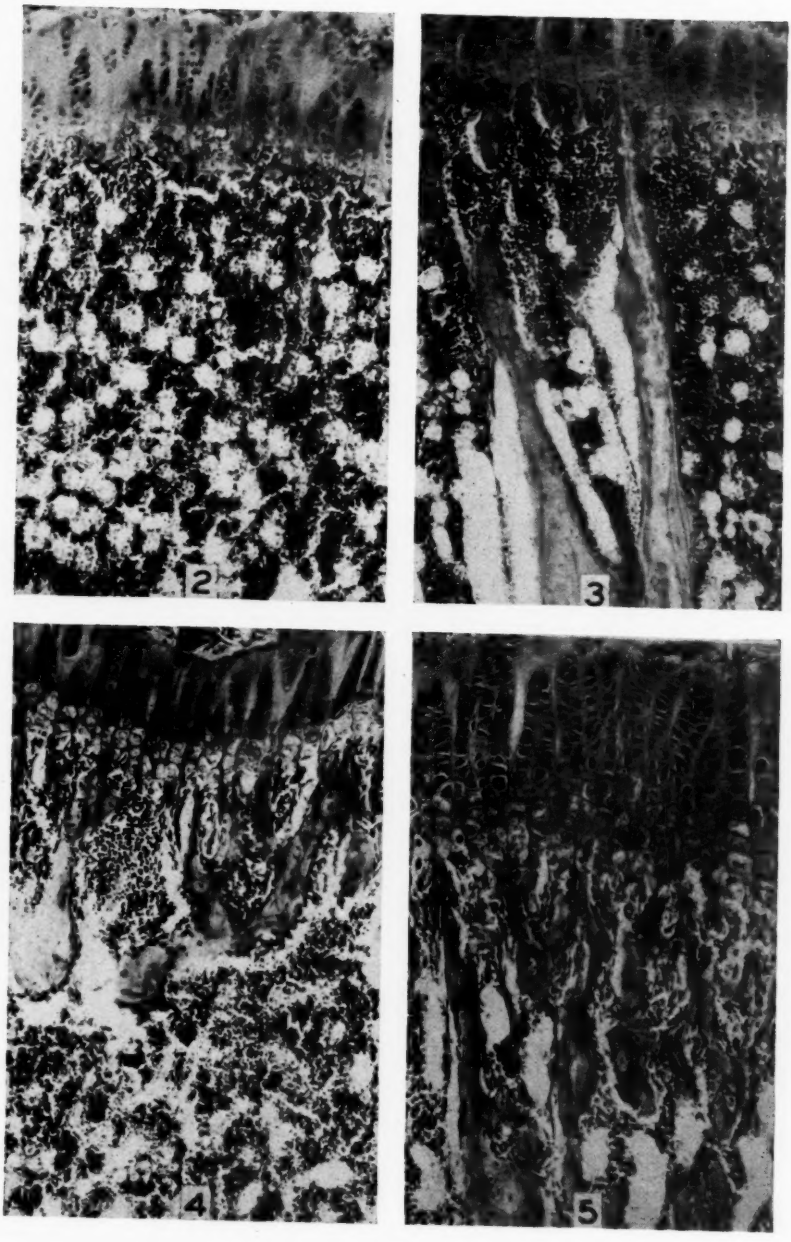

PLATE 1. FIGS. 2–5.

of body weight in the group injected with the solution containing elemental iodine was 7.1 mg. as against 18.8 mg. in the groups receiving iodide only (Table 3). Actually, the injection of a solution containing elemental iodine in high dosage completely inhibited the goiterogenic effect of thiouracil, and thus simulated the action of the thyroxine (Table 3). In contrast to the difference in degree of response to these two types of iodine solutions when they were injected, there was no difference when the same high dosage was fed (Table 3).

The cell height of the thyroid epithelium after thiouracil feeding or pellet implantation is strikingly increased. The cell heights after thiouracil treatment averaged 13.5μ compared with 9.7μ in normal controls (Tables 2 and 3). The colloid of the thyroid was depleted, the epithelium was folded and the number of mitoses was increased (Fig. 10).

All dosages of iodide alone, whether injected or fed, failed to prevent the histological changes which normally result from thiouracil administration (Fig. 11). Nor were the average cell heights significantly lowered by the iodide therapy (Table 3). However, the subcutaneous injection of solutions containing iodide plus elemental iodine in total iodine dosages equal to those used in the groups treated with iodide alone, prevented the depletion of colloid, and significantly reduced cell heights in the thiouracil treated rats (Table 3). The average cell height was lowest in the group treated with the largest dosage of elemental iodine (Table 3, Fig. 12). However, it was not as low as when thyroxine was injected (Table 3, Fig. 13), although it was lower than in normal untreated controls. The oral administration of a solution containing elemental iodine did not reduce the cell height of thiouracil treated rats and, thus, it was as ineffective as the oral administration of equivalent amounts of iodine in the form of iodide (Table 3).

b. Chicks. Chicks fed 0.1% thiouracil for ten days had an average thyroid weight over seven and one-half times that of the controls (Table 4). Iodide injections did not prevent the weight increase. However, when a solution containing similar total amounts of iodine was injected, except that about two-thirds was iodide and one-third elemental iodine, the goiterogenic effect of thiouracil was completely inhibited.

Histological examination of the chick thyroids revealed that thiouracil feeding increased the cell height to almost double that in the

PLATE 2. Sections through the thyroid glands of normal female rats. ×444.

FIG. 6. Untreated control.

FIG. 7. Treated during the last 22 days with daily subcutaneous injections of 8.9 mg. of iodide (I^-).

FIG. 8. Treated during the last 22 days with daily subcutaneous injections of 5.6 mg. of iodide plus 3.3 mg. of elemental iodine (I_2).

FIG. 9. Treated during the last 13 days with daily subcutaneous injections of 7.5 μg. of d,l-thyroxine.

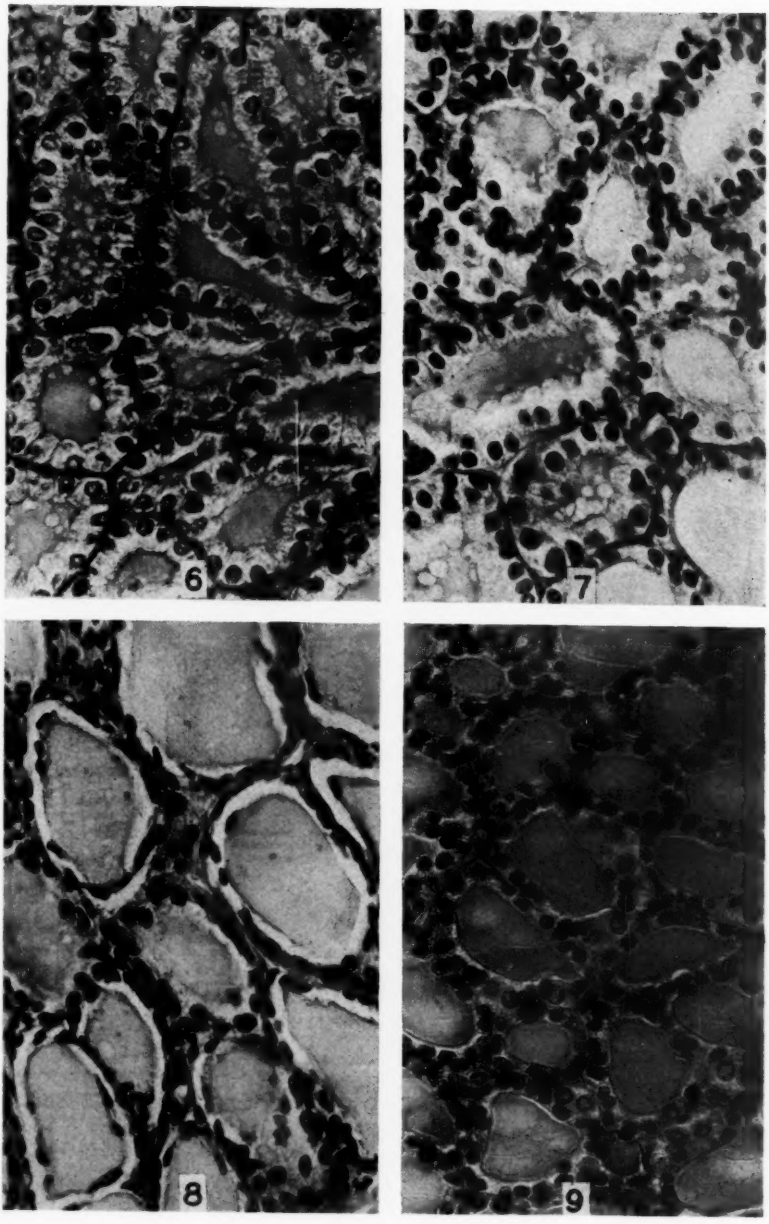

PLATE 2. FIGS. 6–9.

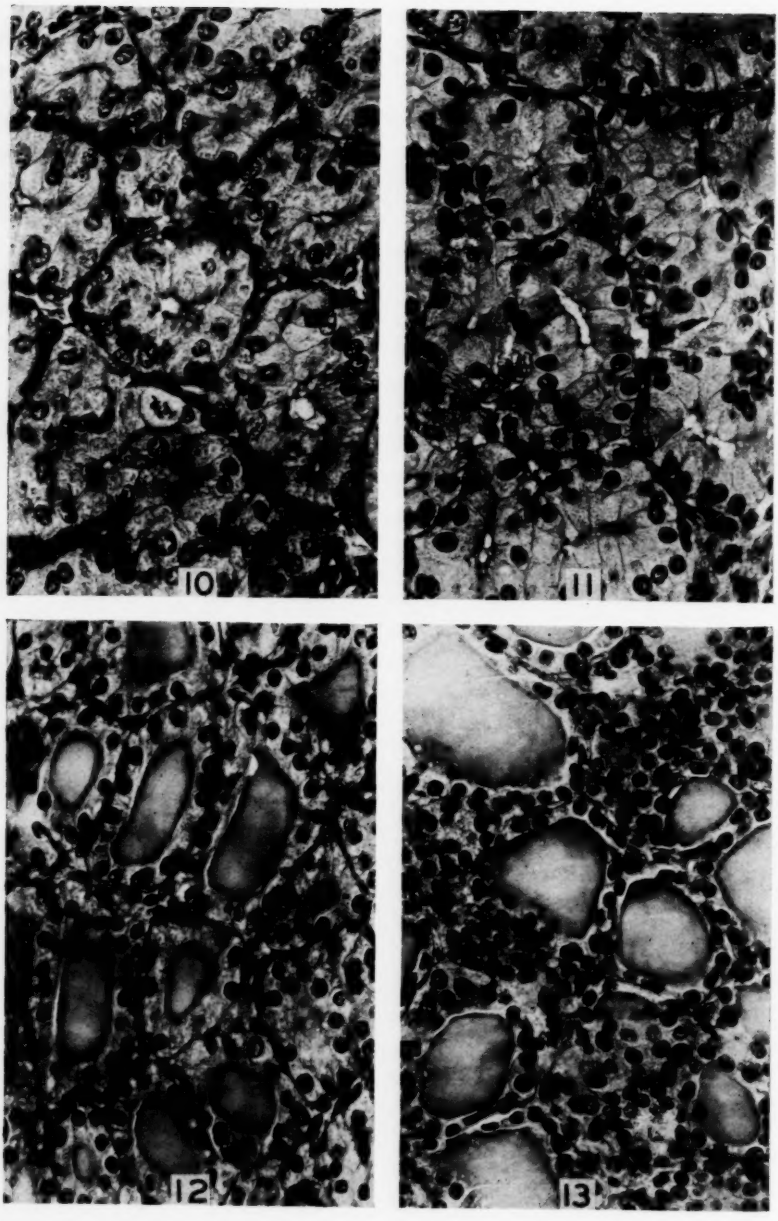

PLATE 3. FIGS. 10–13.

untreated controls (Table 4). The depletion of the colloid, however, was not as striking as in rats. Iodide administration failed to significantly alter the degree of hypertrophy due to thiouracil. However, administration of a solution containing elemental iodine completely inhibited the cell height increase (Table 4).

TABLE 4. THYROID WEIGHT AND THYROID EPITHELIUM CELL HEIGHT OF ONE-DAY OLD CHICKS FED 0.1% THIOURACIL AND TREATED WITH IODIDE (I^-) OR IODIDE PLUS ELEMENTAL IODINE (I_2) BY SUBCUTANEOUS INJECTION FOR 10 DAYS

Treatment		No. of anim.	Thyroid wt., mg./100 gm. body wt.				Cell height—μ			
I^-mg./d.	I_2mg./d.		Av. $\pm\sigma$	Ratio Contr.	P	P_1	Av. $\pm\sigma$	Ratio Contr.	P	P_1
None	None	6	63.5±26.0	7.56	<0.01		7.9±1.0	1.84	<0.01	
5.4	None	6	50.3±21.0	6.00	<0.01		8.3±1.2	1.93	<0.01	
3.4	2.0	6	8.4± 6.2	1.00	>0.09	<0.01	4.6±1.1	1.07	>0.9	<0.01
Untreated Controls		5	8.4± 1.3	1.00			4.3±0.6	1.00		

P = P value of groups compared with untreated controls.
P_1 = P value of group compared with group treated with iodide only.

TABLE 5. THYROID WEIGHT AND CELL HEIGHT OF THYROID EPITHELIUM OF NORMAL YOUNG FEMALE RATS FED 1.0% SULFADIAZINE AND TREATED WITH IODIDE (I^-) OR IODIDE PLUS ELEMENTAL IODINE(I_2) BY SUBCUTANEOUS INJECTION FOR 13 DAYS

Treatment		No. of anim.	Thyroid wt., mg./100 gm. body wt.				Cell height—μ			
I^- mg./d.	I_2 mg./d.		Av. $\pm\sigma$	Ratio diaz. fed. =1.00	P	P_1	Av. $\pm\sigma$	Ratio diaz. fed. =1.00	P	P_1
None	None	3	38.6±6.6	1.00		<0.01	14.8±0.6	1.00		>0.2
8.9	None	4	23.4±4.5	0.61	<0.01		14.3±0.5	0.97	>0.2	
5.6	3.3	4	6.8±1.3	0.18	<0.01	<0.01	5.7±0.0	0.39	<0.01	<0.01

P = P value of groups compared with sulfadiazine fed controls.
P_1 = P value of groups compared with group treated with iodide only.

4. Sulfadiazine Treated Group. Normal young female rats fed a diet containing 1.0% sulfadiazine for 10 days had an average thyroid weight more than five times that in normal controls. As in the thiouracil-induced goiters, injection of iodide casued a significant reduction in this weight (Table 5). However, the injection of a solution containing elemental iodine was strikingly more effective than the iodide and completely prevented the goiterogenic action of sulfadiazine.

Histological examination of these thyroids reveals that iodide injections did not prevent the increase in cell height due to sulfadiazine.

PLATE 3. Sections through the thyroid glands of normal female rats fed 0.1% thiouracil for 13 days before autopsy. ×444.

FIG. 10. Untreated thiouracil-fed control.

FIG. 11. Treated during the last 13 days with daily subcutaneous injections of 8.9 mg. of iodide (I^-).

FIG. 12. Treated during the last 13 days with daily subcutaneous injections of 5.6 mg. of iodide plus 3.3 mg. of elemental iodine (I_2).

FIG. 13. Treated during the last 13 days with daily subcutaneous injections of 7.5 μg. of d,l-thyroxine.

Injections of a solution containing elemental iodine, however, completely prevented it, and an average cell height significantly lower than normal was found (Table 2; Table 5).

The ability of a solution containing elemental iodine, when subcutaneously injected, to prevent the formation of a goiter is not due to a decreased intake of goiterogen. All groups ate essentially similar amounts, and therefore, received similar amounts of the drug. Furthermore, thiouracil dosage was assuredly adequate in the pellet treated groups.

DISCUSSION

The experiments above outlined clearly demonstrate that elemental iodine, injected subcutaneously into the rat or chick, gave effects similar to those produced by thyroxine, whereas the injection of the reduced form, as sodium iodide, gave only slight evidence of thyroxine-like activity. Harington (1944 a, b) has suggested that the essential biochemical reaction in the biosynthesis of thyroxine may be the action of an oxidative system which liberates elemental iodine (I_2) from iodide (I^-), and that the substitution of the iodine in the molecule of an organic compound can only occur after such oxidation. The importance of oxidative mechanisms in the formation of thyroxine in the body, and for the incorporation of iodide into thyroxine *in vitro* has been stressed by numerous investigators (Johnson and Tewkesbury, 1942; Westerfeld and Lowe, 1942; Schachner, Franklin and Chaikoff, 1943; Keston, 1944; Franklin, Chaikoff and Lerner, 1944; Harington and Pitt Rivers, 1945).

The existence of an iodase in the thyroid gland, having the ability to oxidize iodide to elemental iodine, was postulated as early as 1914 by Blum and Grutzner. However, no one has definitely isolated such an enzyme. Peroxidase could not be isolated from the thyroid by Glock (1944) or Astwood (1945). However, oxidase and peroxidase granules are claimed to be demonstrable by histochemical methods in thyroid epithelium (Wegelin, 1909; Graff, 1912; Haeberli, 1916; Okkels, 1934; Dempsey, 1944; De Robertis and Grasso, 1946).

Injections of iodide exerted slight, but definite, growth stimulating effects in the thyroidectomized animals. This may be due to the formation of thyroxine at sites other than the thyroid gland, as suggested by other experiments in which iodide supplements were given to animals on a low iodine diet (Chapman, 1941; Morton, Chaikoff, Reinhardt and Anderson, 1943). However, the growth stimulating effects were much more marked with injections containing elemental iodine.

It is difficult to be certain that the restoration of adrenal weight produced by elemental iodine, but not by iodide, in the thyroidectomized animals was due to the formation of thyroxine-like material. It is possible that the necrosis of the skin produced by the elemental

iodine, but not by the iodide, caused the hypertrophy of the adrenals which is known to occur with non-specific stress in normal animals. However, no adrenal hypertrophy was found in normal or thiouracil or sulfadiazine treated rats injected with solutions containing elemental iodine.

The average cell height of the thyroid epithelium of female rats in our colony is relatively high when compared with some other colonies.[6] Some of the effect of iodine in lowering this cell height may be due to the correction of an iodine deficiency in the diet of our colony. No data are available on the iodine content of the diet. However, the relatively greater ability of injections of solution containing elemental iodine to reduce cell height, when compared with injections of iodide alone, can not be explained solely on the basis of a low iodine diet.

A comparison of the response of thyroid epithelium and thyroid gland weight to iodide treatment reveals that in normal rats cell height is reduced while gland weight is unaltered, whereas in thiouracil treated rats cell height is unaltered while gland weight is reduced. No explanation for this disparity of response can be offered at this time.

Evidence has recently been presented that the reaction of thyrotropic hormone and elemental iodine, but not iodide, *in vitro*, resulted in the formation of an inactive protein iodine complex (Albert, Rawson, Merrill, Lennon and Riddell, 1946). It is conceivable that inactivation of circulating thyrotropic hormone might occur, *in vivo* when a solution containing elemental iodine is injected. This hypothesis could account for the thyroid atrophy in normal animals and the absence of goiter in thiouracil or sulfadiazine treated animals when a solution containing elemental iodine is injected. However, it can not explain the stimulation of growth of thyroidectomized animals by injections of elemental iodine, since it is generally agreed that it is the absence of thyroxine rather than the excess of thyrotropic hormone which slows growth. The experimental results are best explained by the hypothesis that a thyroxine-like material is formed following the injections of a solution containing elemental iodine, although no proof other than physiological is at present available to substantiate this hypothesis.

The mechanism of action of thiouracil is not entirely clarified. However, it is known that thiouracil, although not preventing the immediate uptake of iodide by the thyroid gland (Astwood, 1945; Vanderlaan and Bissell, 1946), does prevent the incorporation of iodide into the hormonal organic iodine of the thyroid (Franklin, Chaikoff and Lerner, 1944; Franklin, Lerner and Chaikoff, 1944).

[6] Cell heights of 3.87μ are reported in Wistar male rats by Uotila (1940), 6.0μ in male rats of unspecified strain by Chapman (1941), and about 4μ to 9μ in Sherman male rats by Cortell and Rawson (1944). In the normal young female rats of our colony studied in this experiment, the thyroid cell height averages 9.7μ.

Astwood (1945) has suggested that the strongly reducing properties of thiouracil might account for its ability to prevent thyroxine formation by preventing oxidation of iodide to elemental iodine, and by so doing prevent the iodination of protein to form thyroxine. He has shown *in vitro* that at neutral pH thiouracil reacts rapidly with elemental iodine to form an iodide and a disulfide compound of thiouracil. It would be possible, consequently, to account for the action of elemental iodine in the present experiment by assuming that it oxidized the thiouracil given, and was itself reduced to iodide, leaving the thyroid gland in a state similar to that when neither iodine nor thiouracil was given. However, 3.3 mg. of elemental iodine a day was able to exert a complete anti-goiterogenic effect against an average of 9.4 mg. of thiouracil per day from pellets. This would place the action of elemental iodine (I_2) outside the range of simple chemical neutralization by mutual oxidation-reduction of elemental iodine and thiouracil. Also, the normal reducing capacity of the subcutaneous tissue probably reduced at least some of the administered elemental iodine. The group of goiterogens to which sulfadiazine belongs does not reduce elemental iodine (Astwood, 1945). Nonetheless, elemental iodine, administered subcutaneously, effectively inhibited the goiterogenic action of sulfadiazine in this experiment.

The effect of oral administration of elemental iodine was tested because it was of interest to determine whether iodination of protein might occur in the gastro-intestinal tract, even though it was known, from *in vitro* studies, that alkaline media were more conducive than acid media to iodination of proteins and thyroxine formation. The acidity of the stomach might possibly impose a barrier. To test this possibility, rats were force fed with a solution containing iodide or with equal amounts of iodine in the form of iodide plus elemental iodine. These solutions were tested in normal and thiouracil-pellet treated animals. Pellets were used in order that the orally administered elemental iodine might not react with simultaneously administered thiouracil. Despite this, no evidence was obtained that the action of orally administered elemental iodine differed from that of iodide.

SUMMARY

The subcutaneous injection of a solution containing elemental iodine (I_2) served to reinitiate bone growth and to increase body length in young female thyroidectomized rats. Equivalent dosage of iodide (I^-) only, exerted minimal growth stimulating effects. The loss in adrenal gland weight which occurred in thyroidectomized animals was prevented by subcutaneously administered elemental iodine but not by iodide.

In normal young female rats, the subcutaneous injection of a solution containing elemental iodine caused a marked decrease in cell

height of the thyroid epithelium, and a decrease in the weight of the thyroid gland. Iodide injections, in equivalent dosage, failed to alter gland weight and only slightly lowered cell height.

In thiouracil fed chicks, and in thiouracil fed or pellet treated or sulfadiazine fed young female rats, the subcutaneous injection of a solution containing elemental iodine caused a marked or complete inhibition of the goiterogen. Iodide injections, in equivalent dosages, failed to decrease the cell height or alter the histological appearance of the goiterous thyroid, but did partially reduce the weight of the gland.

Oral administration of solution containing elemental iodine was no more effective than oral administration of iodide solution in influencing thyroid weight and structure.

The subcutaneous administration of elemental iodine to the rat and chick has a thyroxine-like action.

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THE EFFECT OF INSULIN ON THE TOLERANCE OF NORMAL MALE RATS TO THE OVERFEEDING OF A HIGH CARBOHYDRATE DIET

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INGLE (1946) has reported on the production of alimentary glycosuria by forced feeding in the rat. Large amounts of a high carbohydrate diet were tolerated by each rat, but after the limit of tolerance was exceeded each rat excreted significant amounts of urinary glucose. In the present study it was shown that insulin tended to suppress the amount of glucose excreted by the overfed rat and that larger amounts of the high carbohydrate diet were tolerated before the animal was killed by overfeeding.

METHODS

Male rats of the Sprague-Dawley strain were maintained on Purina Dog Chow until they reached a weight of 300 gm. They were then placed in me-

TABLE 1

Constituent	Grams
Cellu flour (Chicago Dietetic Supply)	120
Osborne and Mendel salt mixture	40
Wheat germ oil	10
Mazola oil	10
Cod liver oil	10
Vitamin K (2-methyl-1,4-naphthoquinone)	100 mg.
Dried yeast (Pabst)	100
Egg albumin (Merck)	160
Starch	500
Dextrin	250
Sucrose	250
Water to make total of	2000 cc.

tabolism cages and maintained on a fluid diet administered by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (1939). The diet was made according to Table 1. During the period of adaptation to forced feeding, the amount of diet was increased gradually to prevent the development of "food-shock." The animals were brought to a full feeding of 26 cc. per rat per day on the fifth day and were maintained at this level, which represents a normal caloric intake for rats

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of this weight, for 7 days. Thereafter, the amount was increased at a certain rate per day (see EXPERIMENTS AND RESULTS) until the death of the animal.

The animals were housed in an air-conditioned room in which the temperature was maintained at 74 to 78 degrees F. and the humidity at 30 to 35 per cent of saturation. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with thymol. Blood glucose was determined by the method of Miller and Van Slyke (1936), and urine glucose by the method of Benedict (1911). Crystalline zinc insulin (Lilly) was administered by subcutaneous injection to the experimental groups immediately following each feeding.

EXPERIMENTS AND RESULTS

Experimental. In Experiment 1 (Fig. 1) 13 rats were overfed by an increment of 2 cc. of diet per day, 7 received 30 units of insulin per day, and 6 remained as untreated controls. In Experiment 2 (Fig. 2)

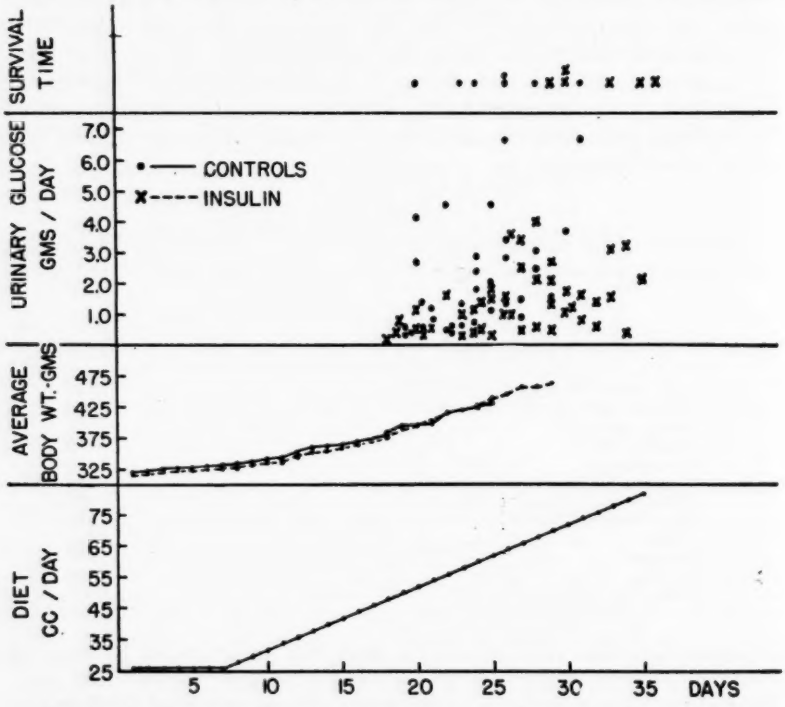

FIG. 1. The effect of insulin upon the tolerance of normal rats to the overfeeding of a high carbohydrate diet. Seven rats received 30 units of crystalline zinc insulin daily and 6 remained as untreated controls.

14 rats were overfed by an increment of 2 cc. per day. Seven animals were treated with 30 units of insulin per day for 7 days, 45 units per day for 7 days, and 60 units per day for the rest of the experiment.

The 7 control animals were untreated. In Experiment 3 (Fig. 3) 13 rats were overfed by an increment of 2 cc. per day until they reached a dietary intake of 52 cc. per day, and thereafter the increment was 1 cc. per day for the remainder of the experiment. Six rats were treated with 30 units of insulin per day for 6 days, 45 units per day

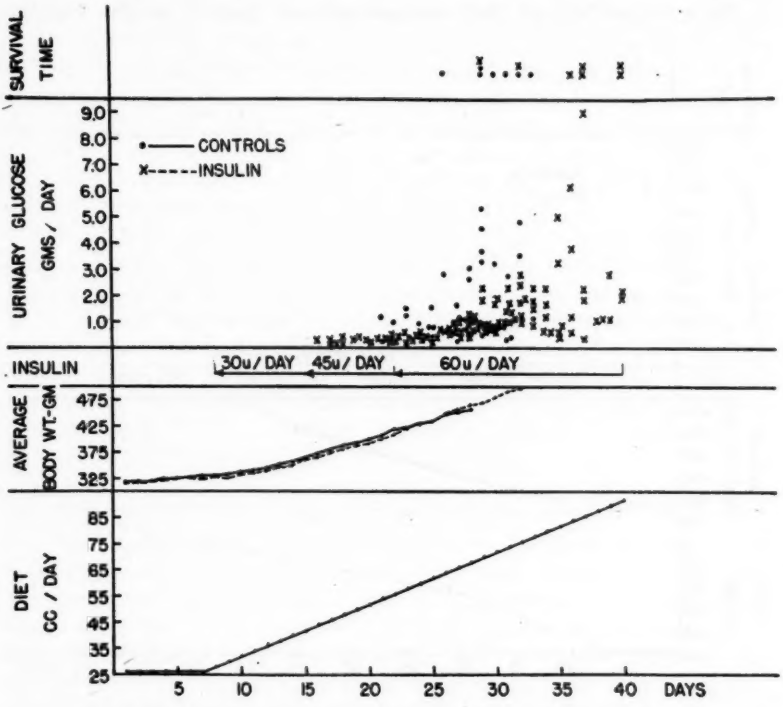

FIG. 2. The effect of insulin upon the tolerance of normal rats to the overfeeding of a high carbohydrate diet. Seven controls and 7 experimentals.

for 6 days, 60 units per day for 12 days, 75 units per day for 12 days, and 90 units per day for the remainder of the experiment. The 7 control animals were untreated.

Urinary glucose. All of the animals were free from glycosuria during the control periods. All of the animals tolerated very large amounts of the high carbohydrate diet before glucose was excreted in the urine. Although there was no tendency for the administration of insulin to delay the onset of alimentary glycosuria, it did definitely suppress the average amount of urinary glucose below that of untreated animals during comparable periods, and it permitted a longer average survival period. At higher levels of food intake, the insulin-treated animals developed a marked glycosuria prior to death.

Blood glucose. A systematic study of blood glucose was not done. In 5 animals which exhibited marked glycosuria during treatment with insulin, it was found that the level of blood glucose was 85 to 129 mgm. per cent prior to the morning feeding but rose rapidly despite treatment with insulin, so that values of 240 to 325 mgm. per cent were found at the end of an hour following feeding.

Body-weight. All of the overfed animals gained weight rapidly.

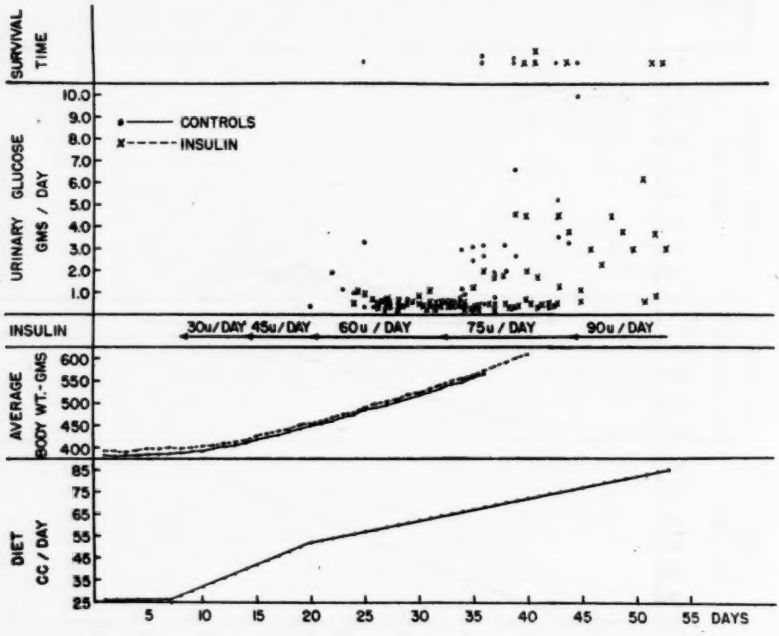

FIG. 3. The effect of insulin upon the tolerance of normal rats to the overfeeding of a high carbohydrate diet. Six controls and 7 experimentals.

There was no significant effect of insulin on the average rates of gain in weight.

Necropsy. The gross pathologic changes were similar to those observed by Ingle (1946) in rats dying with alimentary glycosuria. There was thymus atrophy, adrenal enlargement, regression of the seminal vesicles with some loss of weight by the testes. Animals dying with alimentary glycosuria usually show severe dehydration at the time of death.

DISCUSSION

These data show that treatment with insulin modifies the course of alimentary glycosuria caused by forced feeding in the rat. The average amount of urinary glucose was suppressed below that of the

untreated animal at comparable periods, and the average survival period was prolonged. Insulin did not prevent the development of severe glycosuria during the later stages of overfeeding. There was no evidence that insulin modified the weight changes of these animals to a significant extent.

Our studies on alimentary glycosuria have not touched the numerous problems relating to the damaging effects of overfeeding. What are the changes in structure and in the chemistry of the tissues and body fluids of these animals? Does islet hypertrophy or damage occur? Some of the symptoms shown by rats dying with alimentary glycosuria resemble the symptoms of rats dying from other types of diabetes. How are these symptoms and the causes of death related? The effect of overfeeding in causing regression of the reproductive system is of interest, since starvation causes similar changes. Is this another example of non-specificity in response to damage (Selye, 1937)? In a limited number of studies on overfeeding of high protein and high fat diets, the syndromes have differed somewhat from that caused by the overfeeding of carbohydrate. Our plans for the continued study of overfeeding in this laboratory are principally concerned with the effect of hormones upon the tolerance of intact rats to a high carbohydrate diet and are not likely to include an appraisal of the collateral pathologic changes. The study of the damaging effects of overfeeding is almost a virgin field and deserves attention.

SUMMARY

Three groups of normal male rats were force-fed a high carbohydrate diet at gradual rates of increment until death. One-half of the animals in each group were treated with insulin and the remainder served as untreated controls. Insulin did not delay the onset of glycosuria, but it did depress the amount below the level shown by untreated rats, and it permitted the animals to tolerate larger amounts of carbohydrate before they were killed by overfeeding. There was no significant effect of insulin upon the gains in weight.

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NOTES AND COMMENTS

METABOLISM OF THE ESTROGENS

PRELIMINARY OBSERVATIONS ON THE BIOLOGICAL FATE OF HEXAHYDROEQUILENIN AND ESTRANEDIOL

A PREVIOUS paper from this laboratory (Doisy, Thayer and Van Bruggen, 1942) suggested that dehydrogenation reactions might play a role in the biogenesis of the estrogens, Heard's compound (Δ^5, 7, 9 estratrienol 3-one 17) (Heard, 1941) being suggested as a possible intermediate.

Two compounds[1] that might be biological precursors of the estrogens have been studied. The one, estranediol, represents the completely reduced form of the estrone structure. The other, hexahydroequilenin, (Δ^5, 7, 9 estratriendiol 3, 17) is closely related to Heard's compound.

With both compounds, the primary purpose was to determine if the monkey was able to convert these substances to forms both phenolic and estogenic in nature, the estrogenic activity of the urinary phenolic fractions being used as an index of the degree of conversion.

A small Macacus Rhesus monkey which had been bilaterally ovariectomized sixty days before was used in the first experiment. Control urines were collected in equally spaced intervals through a month's time, each collection being for three days. The animal was injected subcutaneously with a dilute alcoholic suspension of 500 mg. of estranediol. After the injection, all of the urine for a period of thirty days was collected, in four fractions. All the urines were hydrolyzed with 15 vol. % HCl for 10 minutes and after a thorough ether extraction, the phenolic fractions were isolated by standard methods.

Bioassay by a modification of the Marrian method (Thayer, Doisy Jr., and Doisy 1944), showed the presence of less than 4 M.U. of activity per day during the control period. After the injection, on days 1 to 7 the animal excreted 7 M.U. per day; days 8 to 12, 10 M.U. per day; days 13 to 21, 5.5 M.U. per day, and days 22 to 29, 7.0 M.U. per day.

One hundred mg. of the second compound, hexahydroequilenin, in dilute alcoholic suspension, were injected into another monkey previously used in metabolism studies (Doisy, Thayer and Van Bruggen, 1942) but ninety days had elapsed since the injection of the β-estradiol of the previous experiment. A 48 hour control urine showed the presence of 45 M.U. per day, immediately prior to the injection. For the first five days after the injection, the animal excreted 50 M.U. per day while during the next 15 days it excreted 194 M.U. per day. The urine of the next seven days was not assayed. The final four days' collection showed 135 M.U. per day. On the twenty-second day after the injection the animal began to show a bloody discharge from the vaginal orifice. A bleeding of seven days' duration followed. Although the animal had been represented as being hysterectomized and ovariectomized,

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[1] Compounds kindly supplied by Dr. Oliver Kamm, Parke, Davis Co., Detroit, Michigan.

an autopsy conducted two years after the completion of the present experiment showed the presence of a cervix and a uterine stump 3 cm. in length, but the absence of ovaries. However, in the 10 month period preceding this experiment during which the estriol and β-estradiol experiments (Doisy, Thayer and Van Bruggen 1942) had been conducted, no bleeding was ever observed.

Under the influence of the hexahydroequilenin the animal showed some anatomical changes usually elicited by estrogenic compounds, for the skin of the abdominal regions became erythematous and the skin between the eyes became swollen. Since the hexahydroequilenin itself is biologically active only to the extent of 100 M.U. per gram it is doubtful if the parent compound was responsible for the apparent estrogenic response elicited in this animal.

The slight increase in estrogen excretion seen in the first experiment may indicate that the estranediol was not used by the animal to form estrogenic substances. However the degree of biological conversion undergone by a compound when evaluated by the amount of phenolic estrogens excreted may be misleading for only 1 to 20% of injected estrogen can be recovered from the urine of the test animal.

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[3] Since this work has been discontinued and there is no prospect of its resumption, these results are presented for the use of other workers in the field.